PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 38/00, 39/395, C12N 15/00

A1

(11) International Publication Number: **WO 99/18986**

(43) International Publication Date: 22 April 1999 (22.04.99)

(21) International Application Number: PCT/US98/21345

(22) International Filing Date: 9 October 1998 (09.10.98)

(30) Priority Data:

60/063,468	10 October 1997 (10.10.97)	US
08/966,296	7 November 1997 (07.11.97)	US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application

US	08/966,296 (CIP)
Filed on	7 November 1997 (07.11.97)

(71) Applicant *(for all designated States except US)*: THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK [US/US]; West 116th Street and Broadway, New York, NY 10027 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: GOLDBERG, Ira, J. [US/US]; Apartment 11A, 300 West 108th Street, New York, NY 10025 (US). PILLARISETTI, Sivaram [IN/US]; 24 Lincoln Avenue, Bergenfield, NJ 07621 (US).

(74) Agent: WHITE, John, P.; Cooper & Dunham LLP, 1185 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States: AU, CA, JP, MX, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: METHOD FOR INHIBITING THE BINDING OF LOW DENSITY LIPOPROTEIN TO BLOOD VESSEL MATRIX

(57) Abstract

This invention also provides a method of treating atherosclerosis in a subject, comprising administering to the subject an effective amount of a substance capable of binding the amino–terminal region of apolipoprotein B, thereby treating the atherosclerosis. This invention also provides a method for inhibiting the binding of low density lipoprotein to blood vessel matrix in a subject, comprising administering to the subject an effective amount of a substance capable of competing with the amino–terminal region of apolipoprotein B for binding to blood vessel matrix, thereby inhibiting the binding of low density lipoprotein to blood vessel matrix. This invention also provides a method for identifying a compound capable of ameliorating atherosclerosis. Additionally, this invention provides a kit for inhibiting the binding of low density lipoprotein to blood vessel matrix, wherein the kit comprises a substance capable of binding to the amino–terminal region of apolipoprotein B.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

METHOD FOR INHIBITING THE BINDING OF LOW DENSITY LIPOPROTEIN TO BLOOD VESSEL MATRIX

This application is a continuation-in-part of U.S. Serial No. 08/966,296, the content of which is hereby incorporated into this application by reference. This application also claims the benefit of U.S. Provisional application No. 60/063,468 filed October 10, 1997, the content of which is also incorporated into this application by reference.

The invention disclosed herein was made with Government support under NIH Grant Nos. HL 56984, HL 45095 HL 25161 from the Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

Throughout this application, various references are referred to within parentheses. Disclosures of these publications for the first series of experiments in their entireties are hereby incorporated by reference into this application. Disclosures of these publications for the second series of experiments may be found listed at the end of the specification immediately following the Experimental Procedures section and preceding the claims sections to more fully describe the state of the art to which this invention pertains.

Background of the Invention:

Atherosclerosis is the most common cause of coronary heart disease, including angina pectoris and heart attacks. Atherosclerosis is also a major cause of strokes. Atherosclerosis is characterized by a buildup of fatty deposits, called plaques, on the inner walls of arteries and a resulting decrease in the normal flow of the blood through the artery. These plaques are made up of cholesterol and other lipids. Low density lipoproteins

(LDL) are the major carriers of cholesterol in the blood and are believed to be responsible for mediating the arterial accumulation of cholesterol that causes Atherosclerosis. The subendothelial accumulation of LDL is a major atherosclerosis initiating event.

Low density lipoproteins (LDLs) are roughly spherical complexes of lipids and proteins. The predominant protein constituent of LDL is Apolipoprotein B-100 (ApoB100). L. Chan (1992) J. Biol. Chem. **267**, 25621-25624. LDL represents only one of a large class of lipoproteins. Other lipoproteins, in order of increasing density, include: chylomicrons, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and very high-density lipoprotein (VHDL).

Several lipoproteins have also been implicated in the etiology of atherosclerosis. For example, lipoprotein lipase (LpL) has been shown to have multiple interactions with lipoproteins and components of the blood vessel wall. U. Saxena et al. (1992) J. Clin. Invest. **89**, 373-380. In particular, LpL has a lipid-binding domain that binds to VLDLs and LDLs with higher affinity than high-density lipoproteins. S. Choi et al. (1995) J. Biol. Chem. **270**, 8081-8086. LpL is also able to associate with proteoglycans in the subendothelial cell matrix of blood vessels. It's been shown that these interactions allow LpL to increase LDL binding to blood vessels and thereby act as an atherogenic factor. J. C. Rutledge, M. M. Woo, A. A. Rezai, L. K. Curtiss, I. J. Goldberg (1997) *Circulation Research* **80**, 819-828.

The polypeptide components of lipoproteins are called apolipoproteins and are synthesized mainly in liver and intestinal mucosal cells. Apolipoproteins exists in several divisions, including the B Apolipoproteins (ApoB).

ApoB48, which contains 2,152 amino acids, is required for chylomicron formation in the intestine. L. Chan (1992) J. Biol. Chem. **267**, 25621-25624.

ApoB100, which contains 4,536 amino acids, is required for assembly of VLDL in the liver. M. M. Veniant, V. Pierotti, D. Newland, C. M. Cham, D. A. Sanan, R. L. Walzem (1997) *J. Clin. Invest.* **100**, 180-188. Other variants of apoB include apoB23, apoB36, and apoB53. J. Kruezer, A. L. White, T. J. Knott, M-L. Jien, M. Mehrabian, J. Scott, S. G. Young, and M. E. Haberland (1997) *J. Lip. Res.* **38**, 324-342. The amino-terminal sequences of apoB tend to be conserved.

Summary of the Invention

The subject invention provides a method for inhibiting the binding of low density lipoprotein to blood vessel matrix in a subject, comprising administering to the subject an effective amount of a substance capable of binding to the amino-terminal region of apolipoprotein B, thereby inhibiting the binding of low density lipoprotein to blood vessel matrix.

This invention also provides a method of treating atherosclerosis in a subject, comprising administering to the subject an effective amount of a substance capable of binding to the amino-terminal region of apolipoprotein B, thereby treating the atherosclerosis.

This invention also provides a method for inhibiting the binding of low density lipoprotein to blood vessel matrix in a subject, comprising administering to the subject an effective amount of a substance capable of competing with the amino-terminal region of apolipoprotein B for binding to blood vessel matrix, thereby inhibiting the binding of low density lipoprotein to blood vessel matrix.

This invention further provides a method of treating atherosclerosis in a subject, comprising administering to the subject an effective amount of a substance capable of competing with the amino-terminal region of apolipoprotein B for binding to blood vessel matrix, thereby treating the atherosclerosis.

This invention also provides a method for identifying a compound capable of ameliorating atherosclerosis, comprising: (a) contacting the compound with the amino-terminal region of apolipoprotein B under conditions permitting binding between the compound and the amino-

terminal region of apolipoprotein B; (b) detecting specific binding of the compound to the amino-terminal region of apolipoprotein B; and (c) identifying the compound that specifically binds to the amino-terminal region of apolipoprotein B, thereby identifying a compound capable of ameliorating atherosclerosis.

Additionally, this invention provides a kit for inhibiting the binding of low density lipoprotein to blood vessel matrix, wherein the kit comprises a substance capable of binding to the amino-terminal region of apolipoprotein B

This invention further provides a kit for inhibiting the binding of low density lipoprotein to blood vessel matrix, wherein the kit comprises a polypeptide sharing a sequence of at least 6 amino acids, or analogs thereof, with the amino-terminal region of apolipoprotein B.

Additionally, this invention provides a mutant nonhuman organism that overproduces the amino-terminal region of apolipoprotein B.

Brief Description of the Figures

Figure 1

Amino acid sequence of apolipoprotein B17 (an amino-terminal fragment of apolipoprotein B).

Figure 2

Bar graph comparing LDL's binding affinity for heparin with ApoB17's binding affinity for heparin.

Figure 3

Bar graph comparing ApoB48's binding affinity for heparin with ApoB100's binding affinity for heparin.

Figure 4

Bar graph comparing M19's effect on LDL binding to heparin with M47's effect on LDL binding to heparin.

Figure 5

Bar graph comparing M19's effect on LDL binding to subendothelial matrix with M47's effect on LDL binding to subendothelial matrix.

Figure 6

Bar graph comparing M19's effect on LDL binding to matrix proteins with M47's effect on LDL binding to matrix proteins.

Figure 7

Bar graph showing the effect of ApoB17 on LDL binding to subendothelial matrix.

Figure 8

Bar graph showing that ApoB17 releases matrix bound LDL.

Figure 9

Bar graph showing the effect of ApoB17 on LDL accumulation.

Figure 10.

Comparison of heparin affinity of apoB100- and apoB48-containing lipoproteins. Lipoproteins of density 1.019-1.063 grams/ml were isolated from plasma of human apoB100-expressing transgenic mice and apoE knock out mice. The lipoproteins were concentrated by a second ultracentrifugation, dialyzed, and allowed to bind to 3 ml of heparin-affinity gel in buffer containing 0.075 M NaCl. Lipoproteins were eluted with increasing concentrations of NaCl, either step-wise (Panel A, 8 ml of each buffer) or with a gradient (0.075-0.9 M NaCl, Panel B) in the same buffer. The eluted apoB was analyzed by SDS-PAGE and identified by western blotting (A) or Coomassie Blue staining (B). Both apoB48 and B100 eluted at similar salt concentration (~0.26 M NaCl).

Figure 11.

Human LDL and apoB17 binding to heparin. Human LDL were isolated by ultracentrifugation and apoB17 was produced in the medium of 293 cells after infection by an apoB17-producing adenovirus. The medium and human LDL were applied to a heparin-affinity column in 0.075 M NaCl and the column was eluted with buffer containing increasing concentrations of NaCl, as described in figure 1. The eluted apoB was assessed by western blot analysis as described in Methods. Densitometric estimate of the recovery of apoB100-lipoproteins and apoB17 in each fraction after a step elution are shown.

Figure 12.

Antibodies to the NH_2-terminal region but not the carboxyl terminal region of apoB inhibit LDL binding to heparin-agarose:

A. Human LDL was incubated for 1 h with an equimolar amount of monoclonal antibodies to apoB (MB). Heparin-affinity chromatography of human LDL was performed in the presence of MB19, an antibody directed to the NH2-terminal region, or MB47, an antibody that inhibits LDL binding to the LDL receptor. The gel was eluted step-wise with increasing concentrations of NaCl and apoB was assessed by SDS-PAGE and Coomassie Blue staining.

B. Human LDL (200 (g) were incubated with equimolar concentrations of either MBs to the carboxyl-terminal region (4G3-epitope amino acids 2980-3084 and 5E11-epitope amino acids 3441-3569, denoted CTAB), or to the NH_2Bterminal region (1D1-epitope amino acids 474-539, 2D8-epitope amino acids 1438-1481, denoted NTAB) both at equimolar concentrations for 1 h at 4°C. Heparin-agarose chromatography was then performed as described in Figure 1. Fractions were analyzed by SDS-PAGE and stained with Coomassie Blue.

Figure 13

Effect of monoclonal anti-apoB antibodies on LDL binding to subendothelial matrix and dermatan sulfate proteoglycans: Subendothelial matrix (SEM) was prepared from confluent endothelial cells as described in Methods. SEM (panel A) or dermatan sulfate proteoglycan (panel B) containing wells were incubated with ^{125}I-LDL (5 (g/ml) for 1 h in DMEM-1.5% BSA. In the presence or absence of MBs. Unbound LDL was removed and bound LDL was eluted with 0.5 N NaOH and counted.

Figure 14

LDL association with subendothelial matrix in the presence of apoB17.

A. LDL association with subendothelial matrix. This was assessed as described in figure 4. The LDL was added in medium from 293 cells, medium from 293 cells expressing apoB17, and the apoB17-containing medium after immunoprecipitation of the apoB17 (denoted apoB17 anti B).

B. Release of bound LDL by apoB17. In this experiment ^{125}I-LDL was first allowed to bind to subendothelial matrix for 1 h at 37°C. Unbound LDL was removed and matrix containing bound LDL was incubated for 1 h at 37°C with plain medium, or medium obtained from control adenovirus infected 293K cells (293K) or medium from 293K cells infected with apoB17-producing adenovirus (293K B17). Released LDL was counted.

C. Competition with purified proteins. LDL association with subendothelial matrix was assessed as described in figure 4. ^{125}I-LDL was mixed with 20 (g unlabeled LDL (LDL), 1 (g of purified apoB17, or with 10 (g of high molecular weight kininogen (Kg) and added to wells containing subendothelial matrix. Binding was carried out at 37°C for 90 min. Unbound radioactivity was removed and bound radioactivity was extracted with 1N NaOH.

Figure 15.

Competition of LDL association with artery derived decorin by apoB17. Purified decorin was radiolabeled and its association with LDL coated microtiter plates in the presence of increasing concentrations of LDL or apoB17 was assessed.

Detailed Description of the Invention

Apolipoprotein B:

ApoB is the major protein present on plasma lipoproteins LDL, VLDL and chylomicrons. LDL and VLDL contain full-length apoB (B100), whereas chylomicrons contain the amino terminal 48% of the apoB (B48). The complete apoB sequence has been determined (Cladaras et al 1986, EMBO J Vol 5, p3495). Different regions of apoB have been shown to have different functions including binding to LDL receptor and lipids. It was shown that the amino (N) terminal region of apoB (NTAB) can interact with lipase and others have shown that it can interact with macrophage scavenger receptor.

Lipoprotein retention in the blood vessel matrix is considered as a major event in the development of atherosclerosis and we propose that NTAB is the major determinant in mediating LDL binding to different matrix components. Using isolated fragments and antibodies to different regions of apoB, it was shown that domains in the amino terminal 17% of apoB (B17) mediate LDL binding to vessel matrix. B17 has the following amino acid sequence:

MDPPRPALLALLALPALLLLLLAGARAEEEMLENVSLVCPKDATRFKHLRKYTYNYE
AESSSGVPGTADSRSATRINCKVELEVPQLCSFILKTSQCTLKEVYGFNPEGKALLK
KTKNSEEFAAAMSRYELKLAIPEGKQVFLYPEKDEPTYILNIKRGIISALLVPPETE
EAKQVLFLDTVYGNCSTHFTVKTRKGNVATEISTERDLGQCDRFKPIRTGISPLALI
KGMTRPLSTLISSSQSCQYTLDAKRKHVAEAICKEQHLFLPFSYNNKYGMVAQVTQT
LKLEDTPKINSRFFGEGTKKMGLAFESTKSTSPPKQAEAVLKTLQELKKLTISEQNI
QRANLFNKLVTELRGLSDEAVTSLLPQLIEVSSPITLQALVQCGQPQCSTHILQWLK
RVHANPLLIDVVTYLVALIPEPSAQQLREIFNMARDQRSRATLYALSHAVNNYHKTN
PTGTQELLDIANYLMEQIQDDCTGDEDYTYLILRVIGNMGQTMEQLTPELKSSILKC
VQSTKPSLMIQKAAIQALRKMEPKDKDQEVLLQTFLDDASPGDKRLAAYLMLMRSPS
QADINKIVQILPWEQNEQVKNFVASHIANILNSEELDIQDLKKLVKEALKESQLPTV
MDFRKFSRNYQLYKSVSLPSLDPASAKIEGNLIFDPNNYLPKESMLKTTLTAFGFAS

ADLIEIGLEGKGFEPTLEALFGKQGFFPDSVNKALYWVNGQVPDGVSKVLVDHFGYT KDDKHEQDMVNGIMLSVEKLIKDLKSKEVPEARAYLRILGEELGFASLHDLQLLGKL LLMGARTLQGI.

This sequence provides a genetic target to screen individuals at risk for atherosclerosis. This sequence further provides a biochemical target to block the accumulation of LDLs and, thereby, prevent the occurrence of atherosclerosis.

In addition to naturally-occurring forms of polypeptides derived from NTAB, the present invention also embraces other NTAB polypeptides such as polypeptide analogs of NTAB. Such chemical analogs include fragments of NTAB. Following the procedures of the published application by Alton et al. (WO 83/04053), one can readily design and manufacture genes coding for microbial expression of polypeptides having primary conformations which differ from that herein specified for in terms of the identity or location of one or more residues (e.g., substitutions, terminal and intermediate additions and deletions). Alternately, modifications of cDNA and genomic genes can be readily accomplished by well-known site-directed mutagenesis techniques and employed to generate analogs and derivatives of the NTAB polypeptide. Such products share at least one of the biological properties of NTAB but may differ in others.

As examples, products of the invention include those which are foreshortened by e.g., deletions; or those which are more stable to hydrolysis (and, therefore, may have more pronounced or longerlasting effects than naturally-occurring products); or which have been altered to delete or to add one or more potential sites for O-glycosylation and/or N-glycosylation or which have one or more cysteine residues deleted or replaced by e.g., alanine or serine

residues and are potentially more easily isolated in active form from microbial systems; or which have one or more tyrosine residues replaced by phenylalanine and bind more or less readily to target proteins or to receptors on target cells. Also comprehended are polypeptide fragments duplicating only a part of the continuous amino acid sequence or secondary conformations within NTAB, which fragments may possess one property of NTAB and not others. It is noteworthy that activity is not necessary for any one or more of the polypeptides of the invention to have therapeutic utility or utility in other contexts, such as in assays of NTAB antagonism. Competitive antagonists may be quite useful in, for example, cases of overproduction of NTAB.

Of applicability to polypeptide analogs of the invention are reports of the immunological property of synthetic peptides which substantially duplicate the amino acid sequence extant in naturally-occurring proteins, glycoproteins and nucleoproteins. More specifically, relatively low molecular weight polypeptides have been shown to participate in immune reactions which are similar in duration and extent to the immune reactions of physiologically-significant proteins such as viral antigens, polypeptide hormones, and the like. Included among the immune reactions of such polypeptides is the provocation of the formation of specific antibodies in immunologically-active animals [Lerner et al., Cell, 23, 309-310 (1981); Ross et al., Nature, 294, 654-658 (1981); Walter et al., Proc. Natl. Acad. Sci. USA ,78, 4882-4886 (1981); Wong et al., Proc. Natl. Sci. USA, 79, 5322-5326 (1982); Baron et al., Cell, 28, 395-404 (1982); Dressman et al., Nature, 295, 185-160 (1982); and Lerner, Scientific American, 248, 66-74 (1983). See also, Kaiser et al. [Science, 223, 249-255 (1984)] relating to biological and immunological properties of synthetic peptides which

approximately share secondary structures of peptide hormones but may not share their primary structural conformation. These studies show that chemical analogs of NTAB share secondary structual features that make them potentially valuable as competitive inhibitors of LDL binding to subendothelial matrix.

Other chemical analogs of NTAB include peptidomimetic compounds which may be at least partially unnatural. The peptidomimetic compound may be a small molecule mimic of a portion of the amino acid sequence of NTAB. The compound may have increased stability, efficacy, potency and bioavailability by virtue of the mimic. Further, the compound may have decreased toxicity. The peptidomimetic compound may have enhanced mucosal intestinal permeability. The compound may be synthetically prepared. The compound of the present invention may include L-,D- or unnatural amino acids, alpha, alpha-disubstituted amino acids, N-alkyl amino acids, lactic acid (an isoelectronic analog of alanine). The peptide backbone of the compound may have at least one bond replaced with PSI-[CH=CH]. The compound may further include trifluorotyrosine, p-Cl-phenylalanine, p-Br-phenylalanine, poly-L-propargylglycine, poly-D,L-allyl glycine, or poly-L-allyl glycine.

Additionally, the peptidomimetic may have a bond, a peptide backbone or an amino acid component replaced with a suitable mimic. Examples of unnatural amino acids which may be suitable amino acid mimics include β-alanine, L-α-amino butyric acid, L-γ-amino butyric acid, L-α-amino isobutyric acid, L-ε-amino caproic acid, 7-amino heptanoic acid, L-aspartic acid, L-glutamic acid, cysteine (acetamindomethyl), N-ε-Boc-N-α-CBZ-L-lysine, N-ε-Boc-N-α-Fmoc-L-lysine, L-methionine sulfone, L-norleucine, L-norvaline, N-α-Boc-N-δCBZ-L-ornithine, N-δ-Boc-N-α-CBZ-L-ornithine, Boc-p-nitro-L-phenylalanine, Boc-hydroxyproline,

Boc-L-thioproline.

The subject invention provides a method for inhibiting the binding of low density lipoprotein to blood vessel matrix in a subject, comprising administering to the subject an effective amount of a substance capable of binding to the amino-terminal region of apolipoprotein B, thereby inhibiting the binding of low density lipoprotein to blood vessel matrix.

As used herein, the "amino-terminal region of apolipoprotein B" includes sequence shown in Figure 1. The "amino-terminal region of apolipoprotein B" also includes the sequence in Figure 1 wherein amino have been added, deleted or substituted. In an embodiment, the substitutions replace amino acids with other amino acids having similar chemical properties. For example, Valine may be substituted for Leuline. This invention also contemplates using chemical analogs of amino acids. (See previous discussion herein of chemical analogs.)

Further in the practice of this invention, the "administering" may be effected or performed using any of the various methods known to those skilled in the art. In one embodiment, the administering comprises administering intravenously. In another embodiment, the administering comprises administering intramuscularly. In yet another embodiment, the administering comprises administering subcutaneously.

This invention also provides a method of treating atherosclerosis in a subject, comprising administering to the subject an effective amount of a substance capable of binding to the amino-terminal region of apolipoprotein B, thereby treating the atherosclerosis.

In an embodiment of the above-described methods, the amino-terminal region of apolipoprotein B comprises substantially the same sequence as the amino acid sequence shown in Figure 1.

In another embodiment of the above-described methods, the substance is an antibody or a fragment thereof. As used herein, antibody is defined as a polypeptide capable of binding to an antigen. Such polypeptide may have two heavy and light chains or it may be a single-chain antibody. The polypeptide may also include antibody binding domain which fused with another protein.

The fragment of the antibody has binding affinity similar to the antibody. In an embodiment, the fragment of the antibody is produced by proteolytic digestion of the antibody.

In a different embodiment of the above-described methods, the substance is a monoclonal antibody or a fragment thereof.

This invention also provides a method for inhibiting the binding of low density lipoprotein to blood vessel matrix in a subject, comprising administering to the subject an effective amount of a substance capable of competing with the amino-terminal region of apolipoprotein B for binding to blood vessel matrix, thereby inhibiting the binding of low density lipoprotein to blood vessel matrix.

This invention further provides a method of treating atherosclerosis in a subject, comprising administering to the subject an effective amount of a substance capable of competing with the amino-terminal region of apolipoprotein B for binding to blood vessel matrix, thereby treating the atherosclerosis.

In an embodiment of the above-described methods, the substance is the amino-terminal region of apolipoprotein B.

In another embodiment of the above-described methods, the substance is a portion of the amino-terminal region of apolipoprotein B.

The amino-terminal region of apolipoprotein B of is shown in Figure 1. As used herein, "a portion of the amino-terminal region of apolipoprotein B" represents a sufficient part of the amino-terminal region of apolipoprotein B to share secondary structural features with the sequence shown in Figure 1. Such sequences would be recognized by those skilled in the art.

In a different embodiment of the above-described methods, the substance is an amino-terminal fragment of apolipoprotein B designated B17.

As used herein a "fragment of apolipoprotein B designated B17" is a fragment that represents approximately the 17% amino-terminal-most portion of apolipoprotein B.

In yet another embodiment of the above-described methods, the substance comprises a portion of the amino-terminal fragment of apolipoprotein B designated B17.

As used herein, "a portion of the amino-terminal fragment of apolipoprotein B designated B17" a sufficient part of the amino-terminal region of apolipoprotein B to share secondary structural features with the 17% amino-terminal portion of apolipoprotein B.

In still another embodiment of the above-described methods, the substance comprises a chemical analog of the amino-terminal region of apolipoprotein B.

Chemical analogs were described previously described herein.

This invention also provides a method for identifying a compound capable of ameliorating atherosclerosis, comprising: (a) contacting the compound with the amino-terminal region of apolipoprotein B under conditions permitting binding between the compound and the amino-terminal region of apolipoprotein B; (b) detecting specific binding of the compound to the amino-terminal region of apolipoprotein B; and (c) identifying the compound that specifically binds to the amino-terminal region of apolipoprotein B, thereby identifying a compound capable of ameliorating atherosclerosis.

This invention also provides the above-described methods, wherein the compounds are not previously known.

This invention further provides the compounds identified by the above-described methods.

Additionally, this invention provides a method for ameliorating atherosclerosis in a subject comprising administering to the subject an amount of the compounds identified by the above-described methods effective to ameliorate atherosclerosis.

The actual effective amount will be based upon the size of the polypeptide, the biodegradability of the polypeptide, the bioactivity of the polypeptide and the bioavailability of the polypeptide. If the polypeptide does not degrade quickly, is bioavailable and highly active, a smaller amount will be required to be effective. The effective amount will be known to one of skill in the art; it will also be dependent upon the form of the polypeptide, the size of the polypeptide and the bioactivity of the

polypeptide. Variants of the amino-terminal region of apolipoprotein B with a lower affinity for blood vessel matrix will require lower dosages than variants of amino-terminal region of apolipoprotein B with higher affinity for blood vessel matrix. One of skill in the art could routinely perform empirical activity tests to determine the bioactivity in bioassays and thus determine the effective amount.

This invention also provides a pharmaceutical composition comprising an amount of the above-described compounds effective to ameliorate atherosclerosis and a pharmaceutically acceptable carrier.

For the purposes of this invention "pharmaceutically acceptable carriers" means any of the standard pharmaceutical carriers. Examples of suitable carriers are well known in the art and may include, but not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solutions, phosphate buffered saline containing Polysorb 80, water, emulsions such as oil/water emulsion, and various type of wetting agents. Other carriers may also include sterile solutions, tablets, coated tablets, and capsules.

Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium sterate, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well known conventional methods.

Additionally, this invention provides a kit for inhibiting the binding of low density lipoprotein to blood vessel

matrix, wherein the kit comprises a substance capable of binding to the amino-terminal region of apolipoprotein B.

In an embodiment, the substance in the above-described kit is an antibody or a fragment thereof.

This invention further provides a kit for inhibiting the binding of low density lipoprotein to blood vessel matrix, wherein the kit comprises a polypeptide sharing a sequence of at least 6 amino acids, or analogs thereof, with the amino-terminal region of apolipoprotein B.

In an embodiment, sequences of at least 6 amino acids are selected by choosing amino acids that are part of antigenic determinants or other prominent secondary structural features. In a further embodiment, the sequence of 6 amino acids is chosen to overlap the antigenic determinant for the monoclonal antibody designated M17. In another embodiment, the sequence of 6 amino acids is chosen to include a portion of the amino-terminal region of apolipoprotein B that contains a proline.

The mutant nonhuman organism may be produced by generation of a transgenic nonhuman organism which has exogenous DNA encoding the amino-terminal region of apolipoprotein B. In an embodiment, the amino-terminal region of apolipoprotein B is B17.

Additionally, this invention provides a mutant nonhuman organism that overproduces the amino-terminal region of apolipoprotein B.

This invention will be better understood from the examples which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more

fully in the claims which follow thereafter.

FIRST SERIES OF EXPERIMENTS

Experimental Details:

Materials:

Preparation of B17:

ApoB17 was cloned and expressed using a recombinant adeno virus in 293 kidney cells. Cells were incubated with control or B17 containing adeno viruses for 36 hours in DMEM medium containing 2% serum. The virus-containing medium was then removed, and DMEM medium containing 1.5% BSA was added to the cells and incubation was continued for eight hours. During this time B17 was secreted into medium by cells incubated with B17 containing virus but not by control virus. Control and B17 media were collected and filtered. LDL was incubated in these media to assess the binding to matrix or matrix proteins.

Antibodies:

Monoclonal antibody M19 recognizes the amino terminal region of apoB whereas monoclonal antibody M47 recognizes the C-terminal region of apoB (Pillarisetti, et al. 1994 J.Biol. Chem Vol.269:949).

Preparation of vascular matrix:

Normal vascular matrix is composed of several proteins including collagen, laminin, fibronectin and proteoglycans (heparin+heparan, chondroitin and dermatan sulfates). One or more of these proteins may mediate LDL binding to the blood vessel matrix. In vivo this matrix is synthesized by endothelial cells, smooth muscle cells or macrophages. For our in vitro experiments we used a matrix synthesized by endothelial cells. Endothelial cells from bovine aorta and cultured as previously described. (Pillarisetti et al. 1992. Identification of a heparin-releasable lipoprotein lipase binding protein from endothelial cells. *J. Biol.*

Chem. 267: 16517-16522). Briefly cells were grown to confluency in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS). On the day of experiment cells were gently lifted by incubation with saline containing 0.1% Triton X-100+20 mM NH_4OH for 5-10 min. This procedure, as others have shown, removes cells leaving the matrix intact. This matrix contains the above mentioned matrix proteins. LDL binding to this matrix was studied in the presence and absence of B17 or antibodies.

Individual matrix components:

In some experiments, purified and commercially available matrix proteins were used. Collagen, laminin and fibronectin were from Sigma Chemical Co. A gel containing heparin (heparin-Sepharose) was from Pharmacia. Chondroitin sulfate proteoglycan was from Becton and Dickinson. Plastic plates were coated with these proteins and LDL binding to coated plates was studied in the presence and absence of intervening agents.

Blood vessels:

Animal (Golden Syrian Hamster) preparation for injecting LDL into blood vessels was described in detail previously (Rutledge et al Circulation Res. 1997, Vol 80, p819). LDL was injected in the presence or absence of apoB antibodies.

Labeled LDL:

LDL (d<1.063) was isolated from human plasma using density gradient ultracentrifugation. LDL was either radiolabeled with ^{125}Iodine or fluorescent labeled with DiI.

Determining LDL binding:

Two types of experiments were carried out. First, as a model of glycosaminoglycan interaction, LDL binding to heparin Sepharose was performed. Affinity was determined by elution with a step-wise gradient of NaCl. Second,

labeled LDL was incubated in medium containing 3 % albumin with vascular matrix or with purified matrix components (as above) for 1 h at 37°C. Unbound LDL was removed and bound LDL was assessed by releasing with 0.1N NaOH.

Experiments:

NTAB Has a Higher Affinity for Heparin Than for LDL

Heparin-Sepharose chromatography was performed on LDL and ApoB17. Eluted apoB was identified by immunoblotting with a polyclonal apoB antibody. A major portion of bound LDL (~60% of total loaded) was eluted at physiological salt concentration, i.e. 0.15M NaCl. (See Figure 2.) In different experiments about 20-40% of bound LDL was eluted at 0.4M NaCl. Some of B17 was also eluted with 0.15M NaCl. A major peak, however, was eluted at 0.4M NaCl. In addition, 15-20% of bound apoB17 was also eluted at 1.0M NaCl. These data show that NTAB has GAG binding regions and has a higher affinity for heparin than LDL.

ApoB 48's Affinity for Heparin is Greater than ApoB 100's Affinity for Heparin

ApoB 100 particles were isolated from mice that overexpresses human B100. ApoB 48 particles were isolated from apoE null mice. By western blot analysis we confirmed that neither B100 nor B48 particles carried apoE. Like in the pervious experiment, most of the bound LDL eluted at 0.15 and some in 0.4M NaCl buffers. (See Figure 3.) In contraction, about 70% of bound B48 was eluted at 0.4M NaCl. This shows that even when NTAB is present as a part of a lipid particle it still has a higher affinity for GAG.

NTAB is an Important Mediator of LDL Binding to Heparin

To further show that LDL binding to heparin is mediated by NTAB, Heparin-Sepharose chromatography of LDL was performed

in the presence and absence of apoB antibodies. ApoB eluting at difference salt concentrations was identified by immunoblotting. In the absence of any antibodies most of the LDL was eluted at 0.15M NaCl. Addition of M47 did not affect this binding and most LDL still eluted at 0.15M NaCl. (See Figure 4.) However, when M19 was used a portion of apoB did not bind and eluted in the unbound fraction (i.e. 0.075M) . Thus, blocking the NTAB in LDL inhibits LDL's interaction with heparin.

NTAB is an Important Mediator of LDL Binding to Matrix

Incubation in the presence of a control IgG did not affect LDL binding to matrix. M19 inhibited LDL binding to matrix by about 50%. (See Figure 5.) In contrast, M47 inhibited LDL binding by only 10%. Again showing that the amino-terminal region of apoB mediates LDL binding to matrix.

NTAB is an Important Mediator of LDL Binding to Matrix Components

Plates coated with different matrix components were incubated with LDL in the presence of apoB antibodies. M19 again inhibited LDL binding. LDL binding to: fibronectin was inhibited by 48%, to collagen by 23%, and to DSPG by 52%. In contrast, M47 inhibited different bindings by only 10-15%. (See Figure 6.) (These values represent % of values obtained for control incubations that did not receive antibodies.) These data show that NTAB is an important mediator of LDL binding to matrix proteins and proteoglycans.

A Soluble Fragment of NTAB Inhibits LDL Binding to Matrix

LDL was allowed to bind to matrix in the presence of media from control virus transfected 293 cells or B17 virus cells. ApoB17 inhibited LDL binding to matrix by 42%. (See Figure 7.) To determine if this inhibition is due to apoB17, B17 was removed by immunoprecipitation and the

media was then used to inhibit LDL binding. The inhibitory effect of B17 was abolished when B17 was specifically removed.

A Soluble Fragment of NTAB Matrix-Bound LDL

We next tested for B17's ability to release matrix bound LDL. (See Figure 8.) In this experiment LDL was first bound to matrix and then incubated with control or B17 media. Compared to control medium or 293 conditioned media, B17 conditioned medium released ~1.7 fold more LDL into the medium. (See Figure 9.)

In Vivo Effects of Overexpression of apoB17 on Atherosclerosis Development

An adenovirus producing apoB17 in mice was produced. This virus led to detectable amounts of apoB17 in the plasma for more than 30 days after injection of the virus into mice. Three atherosclerosis prone apoE knock out mice received the virus and another three received a control virus. ApoB17 was found in the bloodstream and most of this apoB17 was not associated with circulating lipoproteins. At age 12 weeks, four week after viral injection, the mice were sacrificed and atherosclerosis in the aortic root was quantified. The B17 mice had approximately half the amount of atherosclerosis as the control group (166,000 versus 382,000 um of atherosclerosis). This pilot study suggests that NTAB will be anti-atherogenic.

Further Characterization of the Atherogenic Region of ApoB

In order to more precisely localize the atherogenic regions of apoB, we have constructed a plasmid that produces apoB7, the amino-terminal 7% of apoB. By expressing these and similar constructs containing different fragments of the amino-terminal region of apoB, it will be possible to identify even more precisely the atherogenic region of apoB. Further, by mutating this region, we may render

apoB non-atherogenic.

Summary of Results for the First Series of Experiments:

^{125}I-LDL binding to matrix was inhibited, by approximately 50%, by monoclonal antibody MB19 that recognizes the amino-terminus of apoB, but not by MB47 that recognizes the C-terminus of apoB.

LDL binding was also inhibited by B17 but not control medium. The inhibition caused by B17 was lost following removal of B17 from medium by immunoprecipitation with apoB antibodies.

M19 but not M47 inhibited LDL binding to different matrix components. To chondroitin sulfate by 42%, to heparin by 43% and to laminin by 23%.

^{125}LDL binding to heparin-Sepharose was also inhibited by M19.

LDL binding to blood vessel was inhibited, by >50%, by M19 but not by M47.

Conclusions:

These data show that LDL can interact with different matrix components and NTAB is a significant mediator of these interactions. In particular, our studies show that the amino-terminal region of apoB is an important mediator of LDL binding to the subendothelial matrix of blood vessels. Finally, it is concluded that the NTAB, which is thought to extend away from the core of LDL, causes LDL to accumulate in arteries.

SECOND SERIES OF EXPERIMENTS

Introduction

An initial event in atherosclerosis is the retention of lipoproteins within the intima of the vessel wall. The co-localization of apolipoprotein (apo) B and proteoglycans within lesions has suggested that retention is due to lipoprotein interaction with these highly electronegative glycoconjugates. Both apoB100- and apoB48-containing lipoproteins, i.e. LDL and chylomicron remnants, are atherogenic. This suggests that retention is due to determinants in the initial 48% of apoB. To test this, the interaction of an apoB fragment (B17), and apoB48- and apoB100- containing lipoproteins with heparin, subendothelial matrix and artery wall purified proteoglycans was studied. ApoB100-containing LDL from humans and human apoB transgenic mice and apoB48-containing LDL density lipoproteins from apoE knock mice were used. Despite the lack of the carboxyl-terminal 52% of apoB, the apoB48-LDL bound as well to heparin-affinity gel as apoB100-LDL. An NH_2-terminal fragment containing 17% of full-length apoB was made using a recombinant adenovirus; apoB17 bound to heparin as well as LDL. Monoclonal antibodies against the NH_2-terminal region of apoB decreased apoB100 LDL binding to heparin, whereas antibodies against the LDL receptor-binding region did not alter LDL-heparin interaction. The role of the NH_2-terminal region of apoB in LDL interaction with matrix molecules was also assessed. Media containing apoB17 decreased LDL binding to subendothelial matrix by 42%. Moreover, removal of the apoB17 by immunoprecipitation abrogated the inhibitory effect of these media. Antibodies to the NH_2-terminal region decreased LDL binding to matrix and dermatan sulfate proteoglycans. Purified apoB17 effectively competed for binding of LDL to artery derived decorin and to subendothelial matrix. Thus, despite the presence of

multiple basic amino acids near the LDL receptor-binding domain of LDL, the NH_2-terminal region of apoB is sufficient for the interaction of lipoproteins with glycoconjugates produced by endothelial and smooth muscle cells. The presence of a proteoglycan-binding site in the NH_2-terminal region of apoB may explain why apoB48- and apoB100-containing lipoproteins are equally atherogenic.

The hallmark of the atherosclerotic process, and the characteristic that distinguishes it from other inflammatory processes, is the presence of both intra- and extracellular lipid deposits (1). Despite a large body of data on interactions between lipoproteins and isolated proteins and cultured cells, the processes that lead to the accumulation of these lipoproteins and lipids in the artery wall are not well defined. Two types of lipoproteins have been clearly established as atherogenic. They are the apolipoprotein (apo) B100-containing LDL and the apoB48-containing chylomicron remnants. Human genetic disorders resulting in increased circulating levels of either of these lipoproteins cause premature atherosclerosis (2,3). Moreover, mice have been produced that have elevated blood concentrations of apoB100- or apoB48-lipoproteins and increased plasma levels of either of these particles leads to atherosclerosis development (4,5). Thus, a common and perhaps necessary requirement for atherogenesis is elevated plasma levels of lipoproteins containing at least the NH_2-terminal 48% of apoB.

The observation that elevated levels of apoB are the primary cause of atherosclerosis is supported by pathological data that also provide insights into how these lipoproteins accumulate within the artery. ApoB-containing lipoproteins are major components of the atherosclerotic plaque. Immunohistological studies of blood vessels have

demonstrated the presence of LDL-like particles within the intima (6,7). At least two processes can lead to increased LDL in atherosclerosis-prone regions. These regions could be more permeable to lipoproteins, or components of the artery wall could prevent egress of LDL after crossing the endothelial barrier. In vivo studies of the accumulation of LDL in cholesterol-fed rabbits have suggested that this latter process, termed lipoprotein retention, occurs in atherosclerosis prone areas of the aorta (8).

If LDL become associated with components of the artery, their egress would be prevented and the LDL would be retained in the artery. LDL in atherosclerotic lesions are found in regions that are enriched in proteoglycans (9,10), molecules that contain highly electronegative glycosaminoglycans (GAG). Complexes of apoB-containing lipoproteins and proteoglycans have been purified from atherosclerotic portions of blood vessels (11). In addition, in vitro studies have shown that LDL will associate with proteoglycans (12,13) and that LDL-proteoglycan complexes can be produced under experimental conditions (14). Using heparin binding as a model for how LDL interacts with vessel wall proteoglycans, the prevailing view has been that heparin-binding regions of LDL that are predominantly found near the carboxyl-terminal region of apoB are responsible for LDL retention within arteries (15). This is because small peptides from this region contain multiple basic amino acids and bind more tightly to GAG than peptides from other regions of LDL (15-18). However, apoB48 lipoproteins that do not have this region are atherogenic (5). Therefore if proteoglycan interaction is the initial step in atherosclerosis development there must be additional proteoglycan-binding regions on these apoB48-containing lipoproteins or they must initiate atherosclerosis via an

entirely different mechanism, an unlikely possibility.

The NH_2-terminal region of apoB (NTAB) is a relatively hydrophilic portion of the molecule that contains 7 disulfide bonds and is thought to extend away from the surface of the LDL molecule (19). NTAB encompasses approximately 17% of the apoB and is at least 80 kDa. This region of apoB has been shown to bind to lipoprotein lipase (20), interact with the scavenger receptor (21), and bind to a triglyceride-rich lipoprotein receptor on macrophages (22). Moreover, NTAB is required for the initiation of assembly of apoB-containing lipoproteins in the endoplasmic reticulum (23,24).

In this report, experiments are presented to demonstrate that NTAB binds to heparin affinity gels with an affinity equal to or greater than apoB100-containing LDL. In addition, apoB48-containing lipoproteins were observed to bind to heparin as well as LDL. Moreover, much of the binding of LDL to subendothelial matrix and purified arterial wall proteoglycans was inhibited by a truncated apoB protein containing the NH_2-terminal 17% of apoB. Based on these findings, we propose that NTAB contributes to the atherogenicity of LDL and remnant lipoproteins.

Methods

Lipoprotein isolation and apoB detection: Human and mouse LDL were isolated by sequential ultracentrifugation (25). Plasma was subjected to ultracentrifugation at d<1.019 for 24 hours at 40,000 rpm in a Beckman centrifuge at 10(C as described previously (26). The floating lipoproteins containing VLDL and IDL were removed, d=1.012 buffer was added to the infranatant and the d=1.063 solution was used

for ultracentrifugation as above. For the mouse samples, the floating lipoproteins were re-centrifuged to concentrate and dissociate any remaining apoE. LDL were dialyzed and the protein assessed by the method of Lowry et al. (27). LDL was radioiodinated using iodine monochloride as previously described (28) leading to 100-150 cpm/ng.

To confirm the presence of apoB and to detect any residual apoE, the mouse lipoproteins were subjected to Western blot analysis. SDS-PAGE and western blotting were carried out as described previously (20). Briefly, nitrocellulose strips were incubated with the antibodies (1:500 dilution) for 1 h at room temperature, washed five times with phosphate-buffered saline containing 0.1% Tween 20, and then incubated with the corresponding secondary antibody coupled to horseradish peroxidase. The antibodies used included sheep anti-human polyclonal anti-apoB (Boehringer Mannheim), monoclonal anti-apoB antibodies (MB), and polyclonal anti-apoE antiserum. The peroxidase reaction was developed with ECL Chemiluminescence Kit according to the manufacturer's directions (Amersham Life Sciences Ltd., Buckinghamshire, England).

Monoclonal anti-apoB antibodies (MB): Several monoclonal antibodies that interact with different regions of apoB were used. These antibodies have been characterized previously (29,30). MB47 blocks a region of apoB required for LDL interaction with the LDL receptor. The epitope for MB47 has been localized to amino acids 3429-3453 and 3507-3523. MB19 interacts with an epitope within the first 100 amino acids of apoB (30) and has been used to inhibit lipoprotein lipase interaction with apoB (20). In addition, four commercially available antibodies were used (Ottawa Heart Institute Research Corporation, Ottawa, Canada). These were: 1D1-epitope amino acids 474-539, 2D8-epitope amino acids

1438-1481, 4G3-epitope amino acids 2980-3084 and 5E11-epitope amino acids 3441-3569 (29). For experiments in which the effects of antibodies on LDL interaction with hepain-affinity gel were studied, an equimolar amount of the antibodies and LDL were incubated for 1 h at 4(C prior to chromatography.

Adenovirus production of recombinant apoB17: The recombinant, replication-defective adenovirus Ad.ApoB17 was created in two steps. First the ApoB17 cDNA fragment was cloned into the *Eco*RV and *Sal*I cloning sites of plasmid vector pACE (31). This contains, in order, the first 355 bp from the left end of the adenovirus genome, the CMV immediate early promoter, DNA that encodes splice donor and acceptor sites, cloning sites for the desired gene (in this case ApoB17), DNA encoding a polyA signal sequence from the mouse beta globin gene, and approximately 6 Kbp of adenovirus sequence extending from nt 2966 through nt 9197. The recombinant virus was created in vivo in 293 cells by homologous recombination between the ApoB17-containing vector and plasmid JM17, as described (32). The recombinant virus is replication defective in human cells other than 293 cells, which express adenovirus E1A and E1B. Following transfection of the two plasmids, infectious virus was recovered, the genomes were analyzed to confirm the recombinant structure, and then virus was plaque-purified, all by standard procedures (31). The control virus Ad.CMV was created in similar fashion from pACE lacking the ApoB17 insert.

Confluent 293 cells were infected with pACE-B17 and Ad-CMV at a multiplicity of infection of 100 pfu per cell. After 36 h the media were removed and new, non-serum containing media (DMEM-1.5% BSA) were added to the cells. These media

were collected after 8 h. 0.5 ml of each condition medium was used for immunoprecipitation and secretion of B17 into the medium was confirmed by western blotting.

For some experiments, the apoB17 was removed from the 293 media by immunoprecipitation as follows. ApoB17 containing medium was mixed with a 1/500 volume of polyclonal anti-apoB IgG and incubated at 4(C overnight. The IgG and associated apoB17 were then removed by adding 0.05 volume of 10% protein A Sepharose beads (Pharmacia) and centrifugation at 7,000 rpm for 5 min in a microfuge (Eppendorf Model 5415C). This step was repeated a second time to remove any residual IgG. B17 and IgG removal was confirmed by loss of the immunoreactive band by western blot.

Purification of apoB17: ApoB17 was isolated from medium obtained from adenovirus infected cells. The medium was incubated with heparin-affinity gel (Affigel-Heparin, Bio-Rad, Hercules, CA) for 1 h at 4°C. The gel was washed with Hepes buffer (pH 7.3) containing 0.15 M NaCl and apoB17 was eluted with 0.5 M NaCl in Hepes buffer. The eluted apoB17 was detected by western blot and the fractions with the highest apoB17 content were pooled. B17 was further purified by DEAE-cellulose chromatography. Heparin eluted material was diluted to 0.15 M NaCl and loaded onto a DEAE column. The column was washed and apoB17 was eluted with 0.26 M NaCl in Hepes buffer. ApoB17 was detected by western blotting and the fractions containing apoB17 were pooled

Heparin-affinity chromatography: To assess lipoprotein and apoB binding to heparin, apoB17-containing 293 cell medium or isolated lipoproteins in low salt buffer (0.075 M NaCl,

10mM Hepes, pH 7.4) were incubated with heparin-affinity gel. The gel was first washed with 10mM Hepes, pH 7.4 containing 0.075 M NaCl and 1.5% BSA. 3 ml of gel was then incubated for 2 h at 4(C with either conditioned media from 293 cells infected with pACE-B17, human LDL, mouse LDL from human-B100 transgenic mice, or mouse B48-containing lipoproteins from apoE null mouse. After binding, the heparin gel was packed into Bio-Rad poly-prep columns (0.8 x 4cm), and unbound media were collected. The gel was washed and either eluted sequentially with 2.5 volumes (8 ml) of 10 mM Tris-HCl, pH 7.4, containing 0.15 M, 0.4 M, and then 1 M NaCl or with a gradient (12.5 volumes) of 0.075B0.9 M NaCl. Fractions were either directly analyzed by SDS-PAGE or immunoprecipitated and analyzed by western blotting.

For western blot analysis, immunoprecipitation was carried out as described previously (33). Briefly, fractions collected from the unbound, 0.15 M, 0.4 M, and 1 M NaCl eluates were incubated overnight at 4(C with 0.05 volume of 1:50 dilution of anti-apoB polyclonal antibody, followed by incubation with 0.05 volume of 10% protein A-Sepharose CL-4B beads for 3 h. The beads were washed, and bound proteins were eluted by boiling in 100 (l of sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol) for 5 min. Released proteins were analyzed by 5% SDS-PAGE, followed by western blotting with anti-apoB polyclonal antibody as described above.

In some experiments, ^{125}I-LDL radio-iodinated using iodine monochloride was used. To insure that the labeled LDL interacted with proteoglycans in a similar manner to native LDL, the ^{125}I-LDL preparations were chromatographed using Affigel-heparin. Only LDL that eluted in the same position as unlabeled LDL and was therefore not damaged or oxidized

during the labeling procedure was used for subsequent experiments.

Matrix molecules: Subendothelial matrix was produced from bovine aortic endothelial cells as described previously (34). In brief, confluent monolayers of endothelial cells were washed three times with phosphate buffered saline (PBS) and incubated for 5 min in a solution containing 20 mM NH_4OH and 0.1% Triton X-100 at room temperature. Detached cells were removed by washing three times with PBS followed by three times with MEM containing 3% bovine serum albumin (MEM-BSA). This procedure has been shown to leave the intact SEM attached to the surface of the well.

To assess lipoprotein binding to isolated proteoglycans, 24 well plates (Falcon) were incubated overnight in borate buffer (pH 10) containing 10 (g/ml dermatan sulfate proteoglycans (Collaborative Biomedical Products, Bedford, MA). The unreacted regions of the plates were then blocked by incubating the plates in borate buffer containing 1.5% BSA for 2h at room temperature. ^{125}I-LDL (5 (g/well) in PBS-1.5% BSA (PBS-BSA) were allowed to bind at 37°C for 1h. The plates were then washed three times with PBS-BSA. Bound apoB was extracted with 0.5 N NaOH for 1 h at room temperature and the radioactivity was assessed (35).

Competition between LDL and apoB17 for binding to dermatan sulfate proteoglycans: The role of the NH_2-terminal region of apoB in LDL association with purified arterial wall proteoglycans was assessed. Methods for these experiments are similar to those used in previous studies comparing the binding of different LDL species to proteoglycans (36). Decorin was isolated from human arteries as described (37). For these experiments, apoB17 containing a C-terminal FLAG

epitope (DYKDDDDK) (apoB17F) was used. ApoB17F was produced by infection of Sf9 cells with recombinant baculovirus, followed by anti-FLAG immunoaffinity purification of the Sf9 culture medium. Purified apoB17 was quantified by the Lowry method (27).

Results for the Second Series of Experiments

Comparison of apoB100 and apoB48 LDL association with heparin-gel: To test whether lipoproteins containing apoB100 have higher affinity for heparin than do apoB48-containing lipoproteins, LDL were isolated from human apoB100 expressing and apoE knockout mice. The E knockout particles were used to prevent any confounding effects of apoE in the apoB48 particles on the association with heparin. Human LDL contained almost entirely apoB100 and the particles from the E knockout mice were almost entirely B48 particles. Neither lipoprotein had detectable apoE by western blot.

Both lipoproteins were used for heparin-affinity chromatography and eluted either stepwise with increasing concentrations (10A) or with a gradient of NaCl (10B). Only trace amounts of the apoB100 lipoproteins did not bind to heparin and were present in the 0.075 M NaCl buffer, run through. The western blots of the recovered protein in each eluate are shown in figure 10A. Some of the associated apoB100-LDL was eluted with 0.15 M NaCl, physiologic ionic strength. Additional apB100-LDL remained bound and eluted with 0.4 M NaCl; no further apoB100 LDL was eluted with 1M NaCl. Most apoB48 LDL also associated with heparin in the 0.075 M salt buffer. The elution pattern of this LDL was similar to that of the B100 particles, most of the LDL eluted using 0.15 M NaCl and 0.4 M NaCl. Therefore absence

of the C-terminal region of apoB did not result in decreased heparin binding.

To further compare the elution of these two LDL, 100 (g of both lipoproteins were allowed to bind to heparin-affinity gel at the same time and were eluted with a salt gradient. As shown in figure 10B, both apoB100- and apoB48-LDL bound with similar affinities and eluted at a salt concentration of about 0.25-0.3 M NaCl.

ApoB17 binding to heparin-containing gel: We next tested whether an NH_2-terminal fragment of apoB would directly bind to heparin. ApoB17-containing medium (6 ml) was mixed with an equal volume of 20 mM Tris (pH 7.4) to reduce its ionic strength to 0.075 M NaCl and then was applied to heparin-affinity gel (3 ml). The gel was sequentially washed with increasing ionic strength and the apoB was concentrated by immunoprecipitation. Densitometric scans of the western blots were used to estimate the amount of apoB100 and apoB17 eluted with each NaCl concentration. As shown in figure 11, human LDL, eluted in the identical position to the LDL obtained from the human apoB100 expressing transgenic mice; i.e. production of the lipoproteins in the mouse or human led to similar heparin binding. ApoB17 also associated with heparin. Some apoB17 eluted with 0.15 M NaCl, but the majority of B17 was eluted with 0.4 M NaCl. A small amount of the apoB17, less than 20%, remained and was dissociated with the 1 M NaCl buffer. When eluted with a salt gradient, most apoB17 eluted from the heparin between 0.25 and 0.3 M NaCl (not shown). Therefore apoB17 bound to heparin with a similar affinity to apoB100 and apoB48 LDL. This suggested that the amino-terminal 17% of apoB could mediate apoB100-LDL association with heparin.

Effects of monoclonal antibodies on LDL binding to heparin: Monoclonal antibodies were used to block regions of apoB and prevent their accessibility to heparin. MB47 and 5E11 are antibodies that block LDL interaction with the LDL receptor-binding region of apoB. In the experiment in figure 12A, MB47 was added to apoB100-LDL at a concentration sufficient to inhibit LDL uptake by LDL receptor-upregulated cells (38). In separate experiments, both MB47 and 5E11 antibodies blocked LDL degradation by fibroblasts over 80% (data not shown). Most of the LDL eluted from heparin at 0.15M NaCl. Incubation with MB47 led to no appreciably change in LDL elution from heparin-gel. In contrast, MB19 increased the amount of LDL not associating with heparin and found in the 0.075 M NaCl fraction. Therefore antibodies that block the NH_2-terminal, but not LDL receptor-binding region, decreased LDL binding to heparin. These data support the hypothesis that regions other than the LDL-receptor binding region of apoB can interact with heparin.

A similar experiment was performed with a second set of monoclonal antibodies to the NH_2-terminal and the LDL receptor binding regions of apoB. In this experiment, shown in figure 12B, the antibodies (equimolar concentrations) were allowed to associate with the LDL for 1 h at 4°C and the LDL-antibody complexes were mixed with the heparin-affinity gel and the gel eluted with a continuous gradient from 0.075 to 0.9 M NaCl. As in 12A, the antibody directed against the NH_2-terminal but not the antibody that blocks LDL interaction with the LDL receptor, decreased LDL binding to heparin (Figure 12B). Addition of C-terminal antibodies (4G3 and E11- denoted CTAB) did not alter the elution pattern of LDL from that of Figure 10B. In contrast, in the presence of NTAB antibodies (1D1 and 2D8), LDL was eluted in two peaks in fractions both at a

lower salt concentration than that of untreated LDL (Figure 10B). Therefore two different combinations of monoclonal antibodies gave similar results, implicating the NH_2-terminal region of apoB in LDL-heparin interaction.

Effects of MB19 and MB47 on LDL interaction with matrix proteins: Since the promotion of atherosclerosis is thought to require LDL interaction with matrix proteoglycans, the effects of MBs on LDL association with subendothelial matrix and with isolated proteoglycans were assessed. As shown in figure 13A, MB19 decreased LDL binding to subendothelial matrix by approximately 50%, whereas MB47 had little effect on LDL association with matrix.

Since subendothelial matrix contains a number of proteins that may interact with LDL, immuno-inhibition was assessed using plates that were coated with dermatan sulfate proteoglycans. Shown in figure 13B are the effects of MB19 and 47 on LDL association with dermatan sulfate proteoglycans. MB47 had little effect on LDL binding, but MB19 inhibited LDL interaction with the dermatan sulfate approximately 50%.

Competition between NTAB and LDL for binding to subendothelial matrix: As a further test of the role of NTAB in LDL matrix interaction, radioactive LDL association with matrix was assessed in conditioned medium from 293 cells, and from 293 cells that were infected with apoB17 producing adenovirus. As shown in figure 14A, the apoB17-containing medium reduced LDL binding by 42%. To determine if this effect was specifically due to apoB17 rather than other components of the B17-adenovirus infected medium, the same media were used after removing the apoB17 by immunoprecipitation, denoted B17 anti B in the figure.

The apoB17-depleted medium did not block LDL binding to matrix. In a different experiment ^{125}I-LDL was first allowed to bind to subendothelial matrix and then incubated for 1 h at 37°C with plain medium, or medium obtained from control adenovirus infected 293K cells (293K) or medium from 293K cells infected with apoB17 adenovirus (293K B17). As shown in figure 14B, apoB17 media released greater amounts of LDL. Therefore, soluble NH_2-terminal fragments of NTAB compete with LDL and decrease its association with matrix.

Next the effects of LDL, purified apoB17, and kininogen B, another heparin binding protein (Mr~115,000 Da) B on LDL binding to subendothelial matrix were compared. Both LDL (100 (g, a 20 fold excess) and apoB17 (1 (g, an approximately equimolar amount) inhibited LDL association approximately 50%. Kininogen (10 (g) had no effect. These data suggest that apoB17 was a better competitor than unlabeled LDL for interaction with matrix components including proteoglycans.

ApoB17 competition with LDL for association with decorin: To further confirm that NTAB was responsible for LDL binding to proteoglycans and to assess in a more quantitative manner the abilities of LDL and apoB17 to compete for LDL-proteoglycan association, additional studies were conducted using artery derived decorin. Decorin is the major dermatan sulfate containing proteoglycan of artery. The system employed maximizes the LDL-proteoglycan binding by initially using an interaction binding buffer consisting of 50 mM NaCl, 5 mM calcium, and finally a physiologic buffer of 150mM NaCl. For these studies microtiter plates were coated with LDL and the association of radioiodinated artery derived decorin was assessed in the presence of increasing concentrations of either LDL or apoB17. As expected, LDL effectively and

totally competed for binding of decorin to LDL (Figure 15). When apoB17 was used to compete for decorin binding to LDL, essentially all binding to LDL was inhibited. For both LDL and apoB17 less than 1 pmole was necessary for maximum competition. Three separate experiments were completed to assess binding affinities using molar levels of either apoB17 or LDL required to inhibit 50% of decorin binding to LDL. On the average 0.16 (0.02 pmoles (mean (SEM) of apoB17 and 0.40 (0.03 pmoles of LDL were required. For these experiments, 6 levels of the DYKDDDDK peptide used in purification of apoB17 ranging from 10-300 pmoles had no influence binding decorin to LDL (data not shown). These results indicate that apoB17 binds to artery derived decorin and this binding affinity is significantly ($p<0.05$) greater than the binding affinity of intact LDL particle.

Experimental Discussion of the Second Series of Experiments

A central paradigm for atherogenesis is that the process begins by the retention of lipoproteins on vessel wall proteoglycans. Pathological and biochemical information has supported this thesis. Moreover, studies by several investigators showed that regions of apoB would associate with heparin and other GAG (15-18). However these experiments, using short peptides, implicated regions close to the LDL-receptor binding domain (AA 3359-3367) as the heparin binding, and by inference, atherogenic region of apoB. Thus, a widely held assumption is that this region of apoB causes cholesterol-carrying lipoproteins to become pathologic. Our data suggest that this is not the only portion of apoB that will increase lipoprotein association with GAG.

These data show that NTAB alone or associated with lipoproteins can interact with heparin and matrix proteoglycans. The following data support this. 1) ApoB48 lipoproteins, which do not contain the LDL-receptor binding region, bind as well or better to heparin than apoB100-LDL. 2) Antibodies to NTAB, but not the LDL-receptor binding region, decrease LDL association with heparin. 3) Soluble fragments of NTAB bind to heparin better than LDL. 4) Blocking NTAB with MB or competition with soluble NTAB decreases LDL interaction with subendothelial matrix. 5) ApoB17 competes with LDL for binding to decorin, the major dermatan sulfate proteoglycan of the arteries. Together, these data suggest that the interaction of NTAB with proteoglycans is the basis for retention of at least some apoB-containing lipoproteins within the artery. This assumes that retention is dependent on LDL proteoglycan interactions.

Although apoB100 in LDL may contain a number of heparin-binding regions when peptides of apoB are produced, our data suggest that LDL interaction with heparin involves NTAB. The observation that MB19, the anti-NTAB antibody, inhibited LDL binding to heparin and dermatan sulfate should not be interpreted as evidence for the involvement of a specific epitope in this process. Rather the relatively large size antibody, approximately 150 kDa, probably produced steric hindrance of the smaller, less than 100 kDa NTAB. In contrast, MB47, which inhibits LDL interaction with the LDL receptor, does not mask a region that is essential for LDL-heparin interaction. The competition experiments using soluble apoB17 demonstrate that this protein had heparin binding properties, and are suggestive that apoB17 prevents LDL binding to matrix by competing with a similar region on apoB. The observation that NTAB binds more tightly to heparin than does LDL was not unexpected. We had previously observed that a fragment of apoB was found on the surface of cultured endothelial cells and that this protein was dissociated from the cells by treatment with heparin (20).

Prior theories implicating the LDL-receptor binding region of apoB in LDL retention within arteries were consistent with the biochemical data showing that small peptides near this region bound to heparin. The interpretation of these data required the assumption that this region of apoB was situated on the lipoprotein particles in an identical manner to that in the small soluble peptide fragments that were used in the biochemical studies. Other heparin-binding peptides that were identified in the NTAB had less positive charge and did not bind to heparin with as great an affinity. The extrapolation of these observations to that of LDL requires exposure of the peptide on the LDL surface and tertiary configuration changes that do not mask these

regions or alter their charge density, i.e. by ionic interaction with negative charged amino-acids outside of these peptides. The configuration of NTAB when not associated with lipid or even when on apoB48 could differ from its structure in apoB100. Several groups of investigators using protease digestion have shown that NTAB exposure is increased as VLDL is converted to LDL (40-42). Perhaps, for this reason, some larger VLDL are less atherogenic because they bind more poorly to proteoglycans. Similarly, it may be that NTAB contribution to the atherogenicity of apoB48-lipoproteins may be relatively greater than its role in apoB100 LDL. It should be noted that LDL interactions with proteoglycans are a relatively low affinity process compared with that of other heparin-binding molecules (43). For this reason it has been postulated that "bridging" molecules might be required to mediate this process (44).

Recently, Boren et al. (45) created an apoB molecule in which lysine 3363 was mutated to glutamic acid. LDL containing this mutated apoB had a defect in binding to the dermatan sulfate-containing proteoglycan, biglycan; interaction with heparin and decorin was not reported. These data suggest that multiple sites may be important in LDL binding to different proteoglycans or that this mutation produces alterations in apoB structure outside of the basic amino-acid cluster that was being investigated. Such an effect would not be unprecedented as heparin binding often involves the tertiary structural arrangement of basic amino acids.

Studies of the interactions of other proteins with heparin have shown that mutation of individual amino acids does not necessarily lead to identification of heparin binding

regions. For example, several studies to define the heparin-binding region of lipoprotein lipase showed that mutagenesis of basic residues reduced, but did not eliminate, lipoprotein lipase-heparin interaction (46-48). In addition, mutations of lipoprotein lipase outside of the putative heparin binding regions sometimes also result in molecules with defective heparin binding, presumably because the conformation of the protein is altered. More recent studies using chimeric molecules in which large regions of lipoprotein lipase and hepatic lipase were interchanged have shown that a different region mediates heparin binding (49). Therefore protein interaction with heparin appears to be a complex molecular interaction that, at least in some cases, is not modulated by a single charged amino-acid unless that amino-acid is essential for the conformation of a larger region of the protein.

Demonstrating that NTAB mediates lipoprotein association with matrix proteins allows a more consistent understanding of why apoB-lipoproteins are atherogenic. Previous investigators have postulated that apoB48-lipoproteins required additional proteins for retention. Since most of these lipoproteins are remnants found in the postprandial period or in the plasma of patients with dysbetalipoproteinemia, one hypothesis was that apoE, a well-known heparin-binding protein, mediates proteoglycan interaction. This hypothesis, however, is inconsistent with several recent observations. 1) ApoE knockout mice have severe atherosclerosis; thus the apoB48 remnants are retained within the matrix in the absence of apoE. 2) Production of apoE by macrophages, including those within the arterial wall, is anti-atherogenic (50,51). The anti-atherogenic actions of apoE are exclusive of apoE actions to reduce plasma lipoproteins. Therefore, the hypothesis that apoE promotes atherosclerosis by causing

retention of apoB48 remnants is not tenable.

What are the pathobiological implications of these primarily biochemical observations? 1) They lead to an alteration in our view of lipoprotein atherogenicity and allow a more consistent mechanism that explains why apoB100- and apoB48-lipoproteins are equally atherogenic. 2) By beginning to define the atherogenic portion of lipoproteins, a molecular target for intervention at the level of apoB interaction with artery wall proteins becomes discernable. If the results presented in this manuscript can be extended to studies in animal models of atherosclerosis, the potential exists to seek agents that prevent apoB-matrix protein interactions as preventative and/or therapeutic agents for coronary artery disease.

References for the second series of experiments

1. Guyton, J. R. and K. F. Klemp. 1996. *Arterio. Thromb. Vasc. Biol.* 16:4-11

2. Mahley, R. W., K.H. Weisgraber, T.L. Innerarity, and S.C. Rall, S. C. Jr. 1991. *J. Am. Med. Assoc.* 265:78-83

3. Steiner, G. 1993. *Ann. Med.* 25: 431-435

4. Breslow, JL. *Science.* 1996: 272:685-688

5. Veniant, M.M., V. Pierotti, D. Newland, C.M. Cham, D.A. Sanan. R.L. Walzem, and S.G. Young. 1997. *J. Clin. Invest.* 100:180-188

6. Hoff, H.F. and J.W. Gaubatz. 1977. *Exp. Mol. Pathol.* 26: 214-217.

7. Yomantas, S., V.M. Elner, T. Schaffner, and R.W. Wissler. 1984 *Arch. Pathol. Lab. Med.* 108:374-378

8. Schwenke, D.C., and T.E. Carew. 1989. *Arteriosclerosis.* 9: 908-918.

9. Hoff, H.F., and W.D. Wagner WD. 1986. *Atherosclerosis.* 61:231-236.

10. Wagner, W D. 1985.. *Ann. N.Y. Acad. Sci.* 454:52-68

11. Hoff, H. F., and J.W. Gaubatz. 1982.. *Atherosclerosis* 42:273-297.

12. Iverius, P. H. 1972. *J. Biol. Chem.* 247:2607-2613.

13. Bihari-Varga, M., and M. Vegh. 1967. *Biochim. Biophys. Acta.* 144:202-210.

14. Camejo, G., T. Linden, U. Olsson, O. Wiklund, F. Lopez, and G. Bondjers. *Atherosclerosis* 79:121-129.

15. Hurt-Camejo, E., U. Olsson, O. Wiklund, G. Bondjers, and G. Camejo. 1997. *Arterioscler. Thromb. Vasc. Biol.* 17:1011-1017.

16. Camejo, G, S.O. Olofsson, F. Lopez, P. Carlsson, and G. Bondjers. 1988. *Arterioscler.* 8.:368-377.

17. Cardin, A.D., and R.L. Jackson. 1988. *Adv. Exp. Med. Biol.* 243:157-163.

18. Weisgraber, K.H., and S.C. Rall. 1987. *J Biol Chem.* 262:11097-11103.

19. Segrest, J.P., M.K. Jones, V.K. Mishra, G.M. Anantharamaiah, and D.W. Garber. 1994. *Arterioscleros. Thromb.* 14:1674-1685.

20. Pillarisetti, S., S.Y. Choi, L.K. Curtiss, and I.J. Goldberg. 1994. *J. Biol. Chem.* 269: 9409-9412.

21. Gianturco, S.H., R. Li, R. Song, C.R Reese, D. Stinson, D. K. Strickland and W.A. Bradley. 1995. *Circulation* 92:3313 (Abstract).

22. Kreuzer, J., A.L. White, T.J. Knott, M.L. Jien, M. Mehrabian, J. Scott, S.G. Young, and M.E. Haberland. 1997. *J. Lipid Res.*38: 324-342.

23. Ingram, M.F., and G.S. Shelness. 1997 *J. Biol. Chem.* 272:10279-10286.

24. Gretch, D.G, S.L. Sturley, L. Wang, B.A. Lipton, A. Dunning, K.A. Grunwald, J.R. Wetterau, Z. Yao, P. Talmud, and A.D. Attie. 1996. *J. Biol. Chem.* 271:8682-8691.

25. Havel, R.J., H.A. Eder, and J.H. Bragdon. 1955. *J. Clin. Invest.* 34:1345-1353.

26. Goldberg, I. J., N.A. Le, J.R. Paterniti, H.N. Ginsberg, F.T. Lindgren, and W.V. Brown. 1982. *J. Clin. Invest.* 70:1184-1192.

27. Lowry, O. H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. *J. Biol. Chem.* 193:265-275

28. Shepherd, J., K. Bedford, and H. G. Morgan. 1976. *Clin. Chim. Acta.* 66:97-101.

29. Pease, R.J., R.W. Milne, W.K. Jessup, A. Law, P. Provost, J.C. Fruchart, R.T. Dean, Y.L. Marcel, and J. Scott. 1990. *J. Biol. Chem.* 265:553-568

30. Young, S.G., S.J. Bertics, L.K. Curtiss, D.C. Casal, and J.L. Witztum. 1986. *Proc. Natl. Acad. Sci. U S A.* 83:1101-1105

31. Young, C. S. H., Nicolás, A. L., Lu, H., and Munz, P. L. 1998. *In:* Methods in Molecular Medicine, XX: Adenovirus Methods and Protocols Ed.: W. S. M. Wold, Humana Press, Totowa, NJ (In press).

32. McGrory, W. J., Bautista, D. S., and Graham, F. L.

1988. *Virology* 163: 614-617.

33. Pillarisetti, S., T. Vanni-Reyes and I.J. Goldberg. 1996. *J. Biol. Chem.* 271: 15261-15266.

34. Pillarisetti, S., J. C. Obunike, and I. J. Goldberg. 1995. *J. Biol. Chem.* 270:29760-29765.

35. Pillarisetti, S., L. Paka, J.C. Obunike, L. Berglund, and I.J. Goldberg, I.J. 1997. *J. Clin. Invest.* 100: 867-874.

36. Wagner, W. D, Edwards, I. J, St Clair, R.W., Barakat, H. 1989. *Atherosclerosis* 75: 49-59.

37. Register T, C., Wagner W, D., Robbins R, A., Lively, M. O. 1993. Atherosclerosis 98: 99-111.

38. Obunike, J., I. Edwards, S. Rumsey, L. Curtiss, W. Wagner, R. Deckelbaum and I.J. Goldberg. 1994. *J. Biol. Chem.* 269: 13129-13135.

39. Register, T.C., W.D. Wagner, R.A. Robbins, and M.O. Lively. 1993. *Atherosclerosis.* 98:99-111.

40. Chen, G.C., S. Zhu, D.A. Hardman, J.W. Schilling, K. Lau, and J.P. Kane. 1989. *J. Biol. Chem.* 264:14369-14375.

41. Chen, G.C., K. Lau, R.L. Hamilton, and J.P. Kane. 1991. *J. Biol. Chem.* 266:12581-12587.

42. Schonfeld, G., M.J. Tikkanen, and K.S. Hahm. 1985. Adv. *Exp. Med. Biol.* 183:135-157.

43. Conrad, H. E. 1998. *in* Heparin-binding proteins.

(Academic Press. San Diego) p 402-404.

44. Goldberg, I. J. 1996. *J. Lipid Res.* 37:693-707.

45. Boren, J, Olin, K. Lee, I, Chait, A, Wight, T. N, Innerarity, T. L. 1988. *J. Clin. Invest.* 101:2658-2664.

46. Ma, Y., H. E. Henderson, M. S. Liu, H. Zhang, I. J. Forsythe, I. Clarke-Lewis, Hayden, M. R and Brunzell J. D. 1994. *J. Lipid Res.* 35:2049-2059.

47. Hata, A., D.N. Ridinger, S. Sutherland, M. Emi, Z. Shuhua, R.L. Myers, K. Ren, T. Cheng, I. Inoue, and D.E. Wilson. 1993. *J. Biol. Chem.* 268:8447-8457.

48. Berryman, D.E. and A. Bensadoun. 1993. *J. Biol. Chem.* 268: 3272-3276.

49. Wong, H, R.C. Davis, J. Nikazy, K.E. Seebart, and M.C. 50. Schotz. 1991. *Proc. Natl. Acad. Sci. U. S. A.* 88:11290-11294.

50. Davis, R.C., H. Wong, J. Nikazy, K. Wang, Q. Han, and M.C. Schotz. 1992 *J. Biol. Chem.* 267:21499-21504.

51. Fazio, S., V.R. Babaev, A.B. Murray, A.H. Hasty, K.J. Carter, L.A. Gleaves, J.B. Atkinson, and M.F. Linton. 1997. *Proc. Natl. Acad. Sci. U.S.A.* 94: 4647-4652.

52. Bellosta, S., R.W. Mahley, D.A. Sanan, J. Murata, D.L. Newland, J.M. Taylor, and R.E. Pitas. 1995. *J. Clin. Invest.* 96:2170-2179.

What is claimed is:

1. A method for inhibiting the binding of low density lipoprotein to blood vessel matrix in a subject, comprising administering to the subject an effective amount of a substance capable of binding to the amino-terminal region of apolipoprotein B, thereby inhibiting the binding of low density lipoprotein to blood vessel matrix.

2. A method of treating atherosclerosis in a subject, comprising administering to the subject an effective amount of a substance capable of binding to the amino-terminal region of apolipoprotein B, thereby treating the atherosclerosis.

3. The method of claim 1, wherein the amino-terminal region of apolipoprotein B comprises substantially the same sequence as the amino acid sequence shown in Figure 1.

4. The method of claim 1, wherein the substance is an antibody or a fragment thereof.

5. The method of claim 1, wherein the substance is a monoclonal antibody or a fragment thereof.

6. A method for inhibiting the binding of low density lipoprotein to blood vessel matrix in a subject, comprising administering to the subject an effective amount of a substance capable of competing with the amino-terminal region of apolipoprotein B for binding to blood vessel matrix, thereby inhibiting the binding of low density lipoprotein to blood vessel matrix.

7. A method of treating atherosclerosis in a subject,

comprising administering to the subject an effective amount of a substance capable of competing with the amino-terminal region of apolipoprotein B for binding to blood vessel matrix, thereby treating the atherosclerosis.

8. The method of claim 6, wherein the substance is the amino-terminal region of apolipoprotein B.

9. The method of claim 6, wherein the substance is a portion of the amino-terminal region of apolipoprotein B.

10. The method of claim 6, wherein the substance is an amino-terminal fragment of apolipoprotein B designated B17.

11. The method of claim 6, wherein the substance comprises a portion of the amino-terminal fragment of apolipoprotein B designated B17.

12. The method of claim 6, wherein the substance comprises a chemical analog of the amino-terminal region of apolipoprotein B.

13. A method for identifying a compound capable of ameliorating atherosclerosis, comprising:

a. contacting the compound with the amino-terminal region of apolipoprotein B under conditions permitting binding between the compound and the amino-terminal region of apolipoprotein B;

b. detecting specific binding of the compound to the amino-terminal region of apolipoprotein B; and

c. identifying the compound that specifically binds to the amino-terminal region of apolipoprotein B, thereby identifying a

compound capable of ameliorating atherosclerosis.

14. The method of claim 13, wherein the compound is not previously known.

15. The compound identified by the method of claim 14.

16. A method for ameliorating atherosclerosis in a subject comprising administering to the subject an amount of the compound identified by the method of claim 13 effective to ameliorate atherosclerosis.

17. A pharmaceutical composition comprising an amount of the compound identified by the method of claim 13 effective to ameliorate atherosclerosis and a pharmaceutically acceptable carrier.

18. A kit for inhibiting the binding of low density lipoprotein to blood vessel matrix, wherein the kit comprises a substance capable of binding to the amino-terminal region of apolipoprotein B

19. The kit of claim 18, wherein the substance is an antibody or a fragment thereof.

20. A kit for inhibiting the binding of low density lipoprotein to blood vessel matrix, wherein the kit comprises a polypeptide sharing a sequence of at least 6 amino acids, or analogs thereof, with the amino-terminal region of apolipoprotein B.

21. A mutant nonhuman organism that overproduces the amino-terminal region of apolipoprotein B.

FIG. 1

MDPPRPALLALLALPALLLLLLAGARAEEEMLENVSLVCPKDATRFKHLRKYTYNYEAESSS
GVPGTADSRSATRINCKVELEVPQLCSFILKTSQCTLKEVYGFNPEGKALLKKTKNSEEFAA
AMSRYELKLAIPEGKQVFLYPEKDEPTYILNIKRGIISALLVPPETEEAKQVLFLDTVYGNC
STHFTVKTRKGNVATEISTERDLGQCDRFKPIRTGISPLALIKGMTRPLSTLISSSQSCQYT
LDAKRKHVAEAICKEQHLFLPFSYNNKYGMVAQVTQTLKLEDTPKINSRFFGEGTKKMGLAF
ESTKSTSPPKQAEAVLKTLQELKKLTISEQNIQRANLFNKLVTELRGLSDEAVTSLLPQLIE
VSSPITLQALVQCGQPQCSTHILQWLKRVHANPLLIDVVTYLVALIPEPSAQQLREIFNMAR
DQRSRATLYALSHAVNNYHKTNPTGTQELLDIANYLMEQIQDDCTGDEDYTYLILRVIGNMG
QTMEQLTPELKSSILKCVQSTKPSLMIQKAAIQALRKMEPKDKDQEVLLQTFLDDASPGDKR
LAAYLMLMRSPSQADINKIVQILPWEQNEQVKNFVASHIANILNSEELDIQDLKKLVKEALK
ESQLPTVMDFRKFSRNYQLYKSVSLPSLDPASAKIEGNLIFDPNNYLPKESMLKTTLTAFGF
ASADLIEIGLEGKGFEPTLEALFGKQGFFPDSVNKALYWVNGQVPDGVSKVLVDHFGYTKDD
KHEQDMVNGIMLSVEKLIKDLKSKEVPEARAYLRILGEELGFASLHDLQLLGKLLLMGARTL
QGI

FIG. 2A

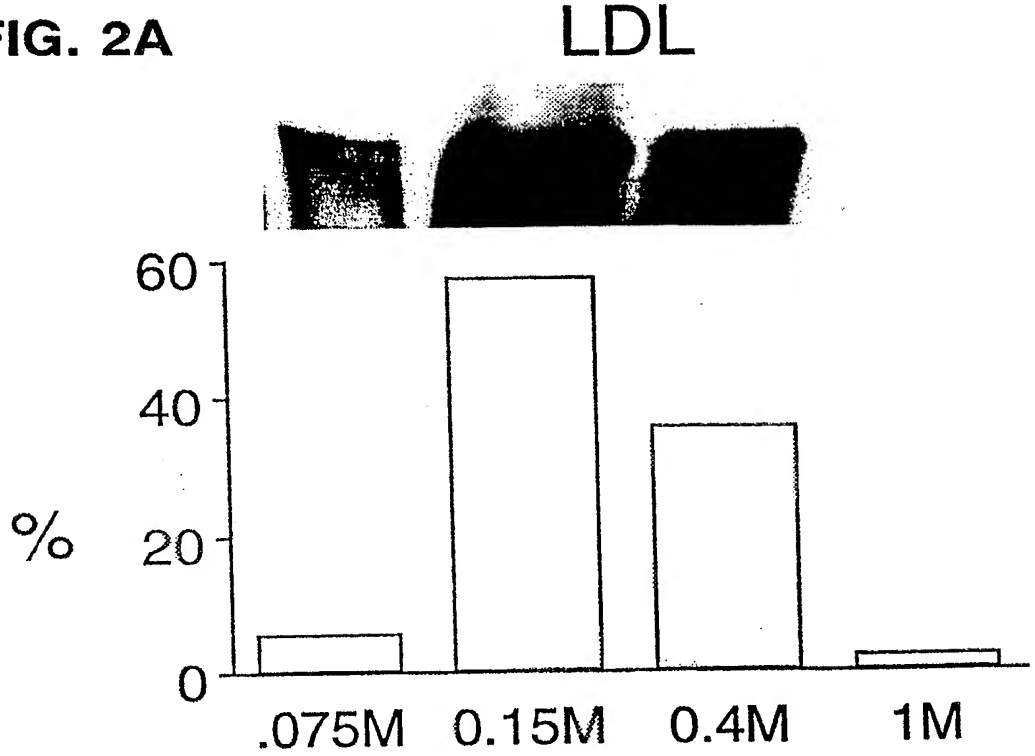

LDL
60
40
20
0
%
.075M
0.15M
0.4M
1M

FIG. 2B

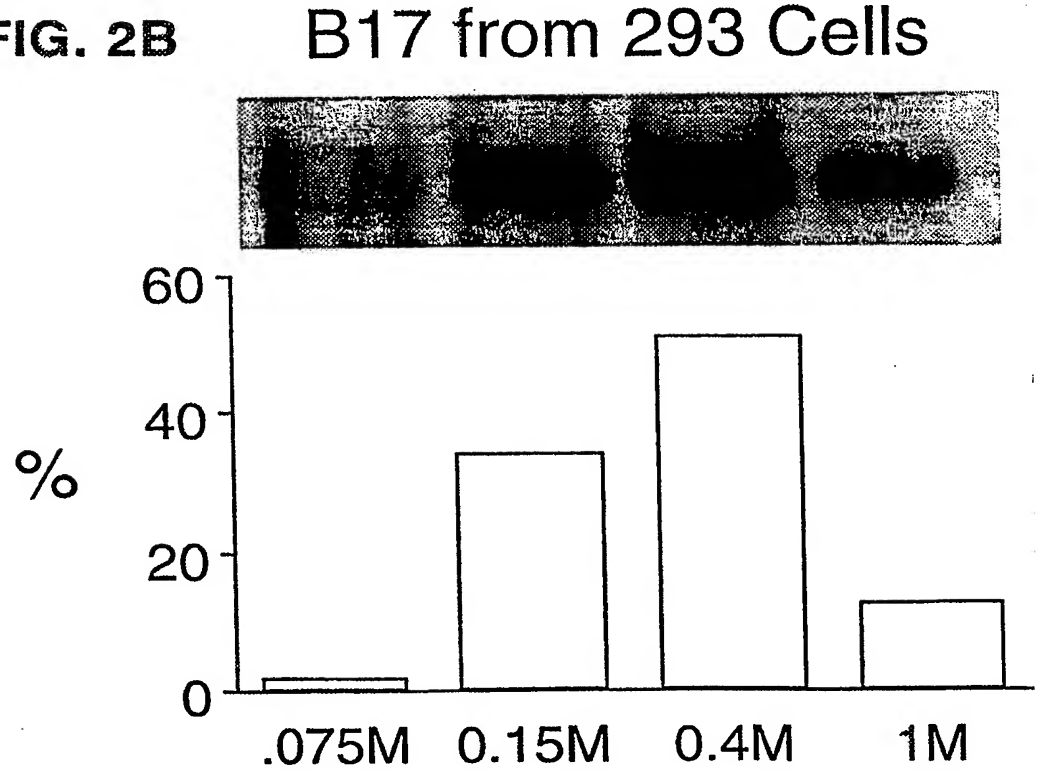

B17 from 293 Cells
60
40
20
0
%
.075M
0.15M
0.4M
1M

FIG. 3A B-100 (ApoB100 Tg Mouse)

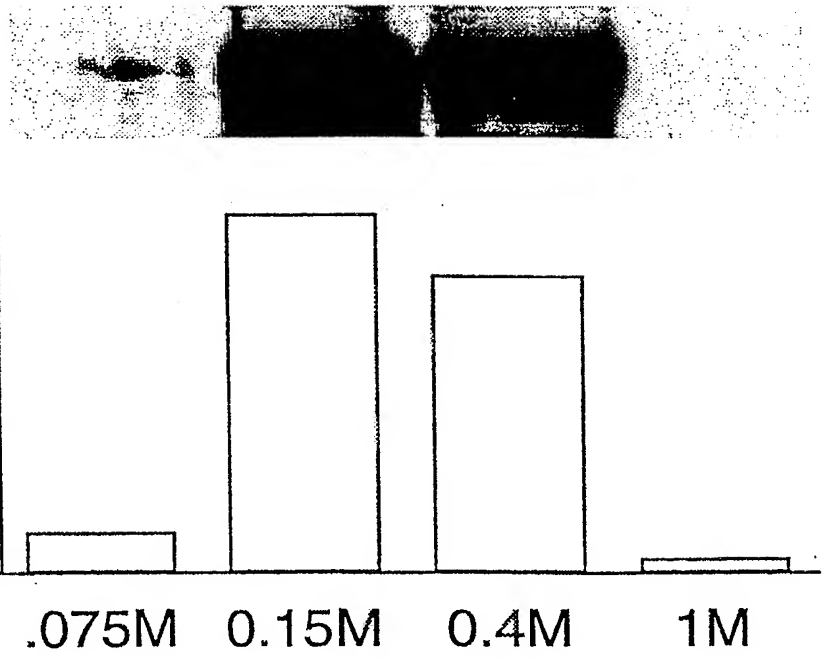


FIG. 3B ApoB48 (E Null Mouse)

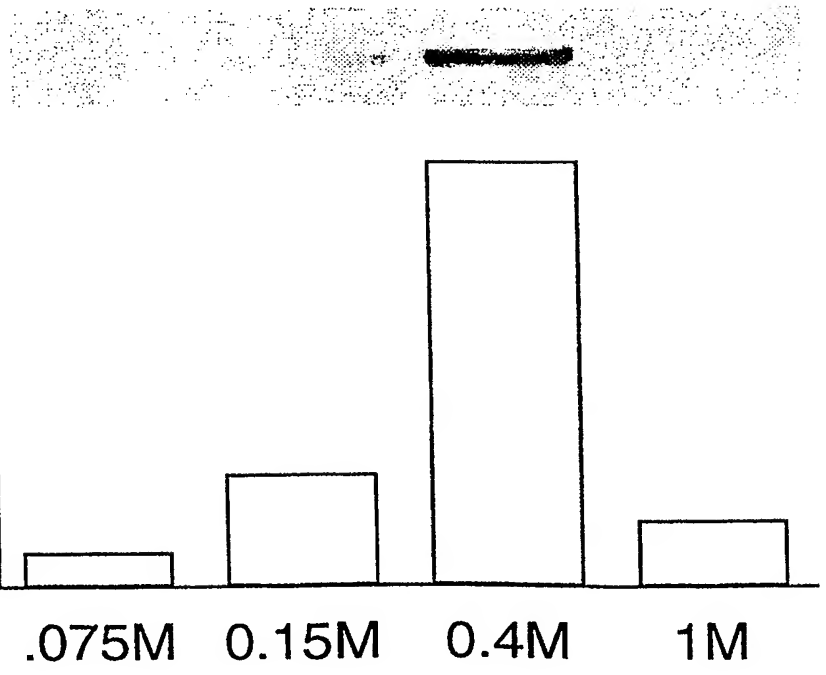


FIG. 4

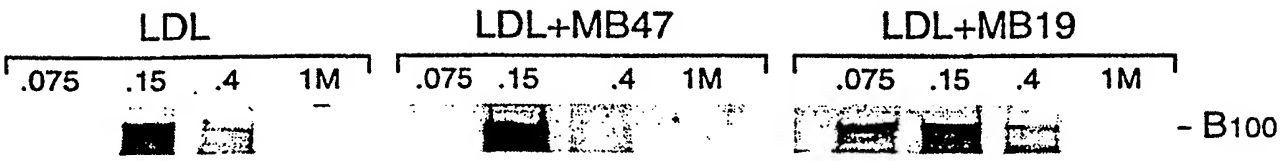


FIG. 5

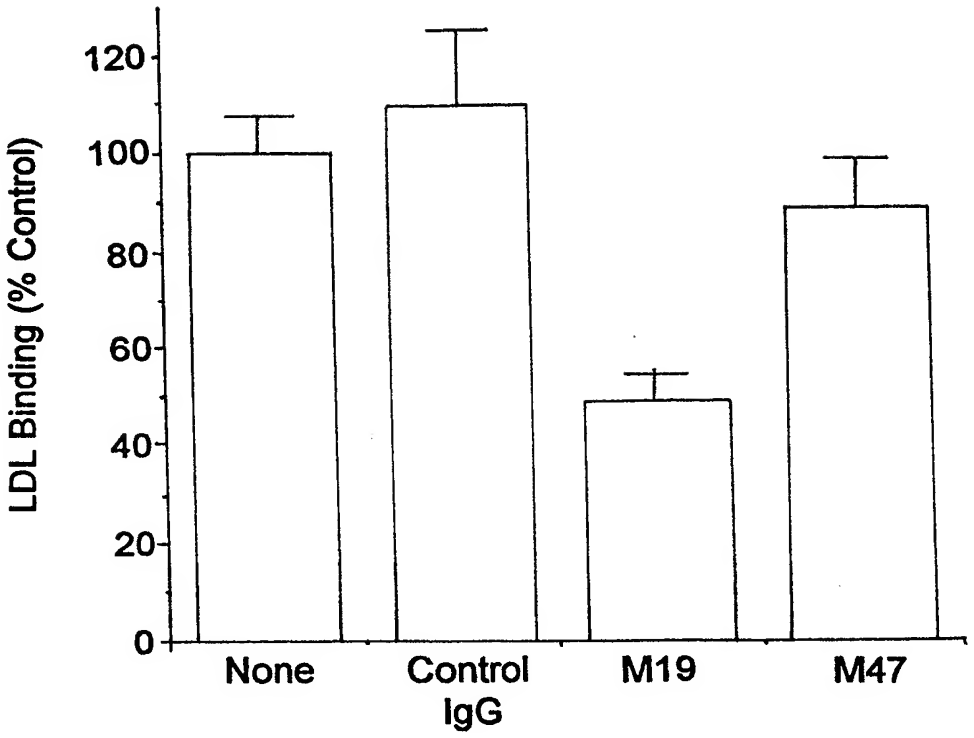

LDL Binding (% Control)
120
100
80
60
40
20
0
None
Control IgG
M19
M47

FIG. 6

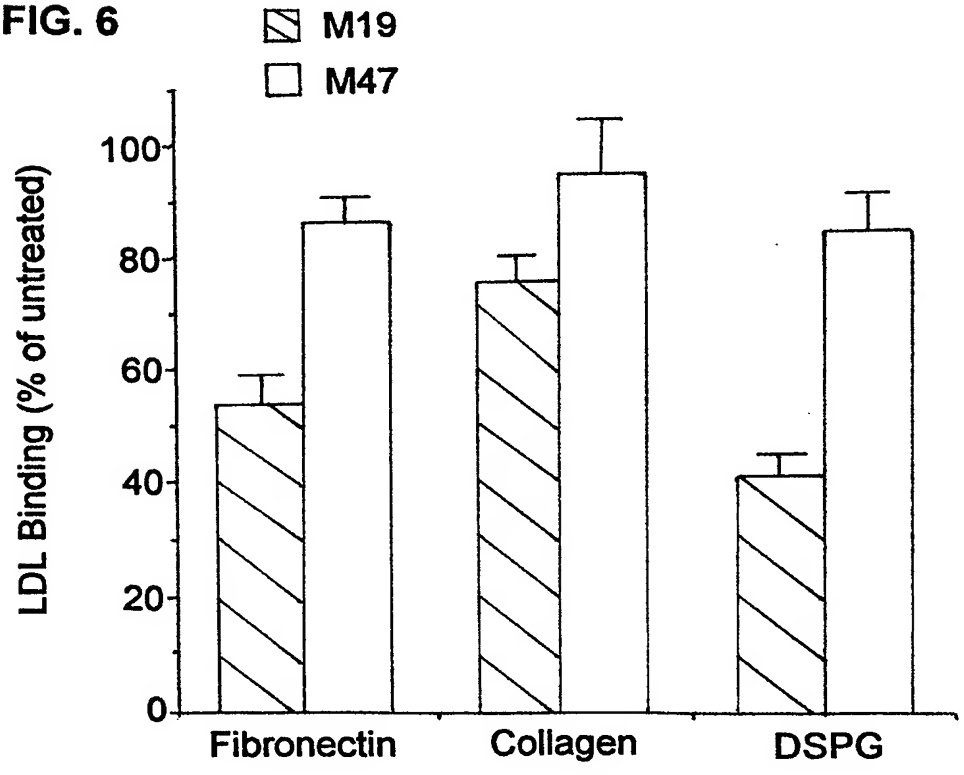

M19
M47
LDL Binding (% of untreated)
100
80
60
40
20
0
Fibronectin
Collagen
DSPG

FIG. 7

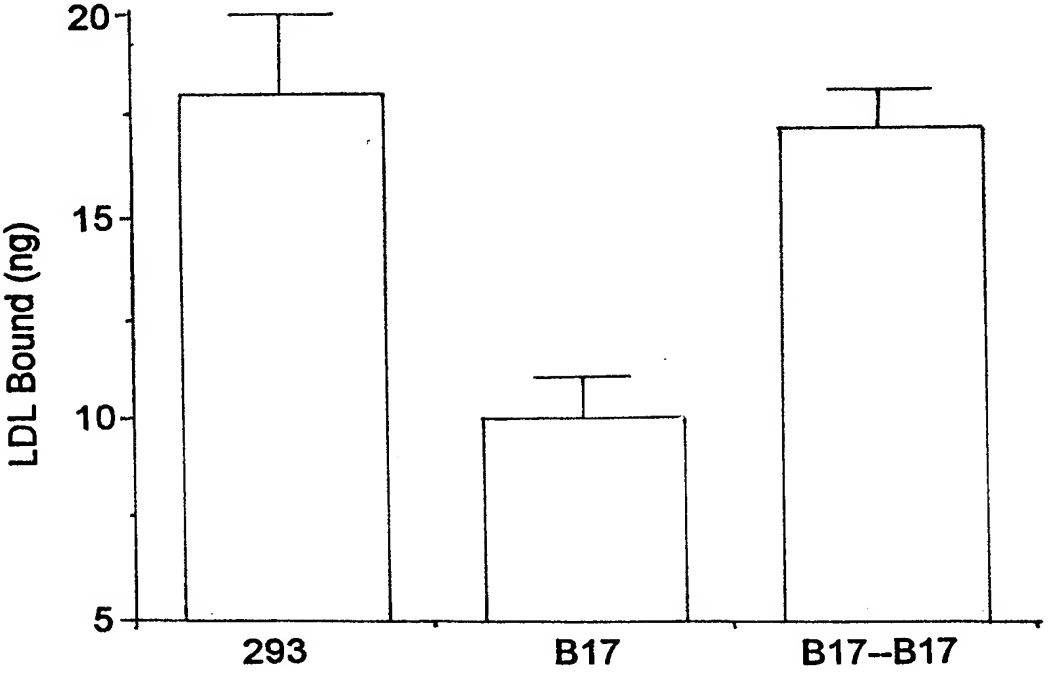

20
15
10
5
LDL Bound (ng)
293
B17
B17--B17

FIG. 8

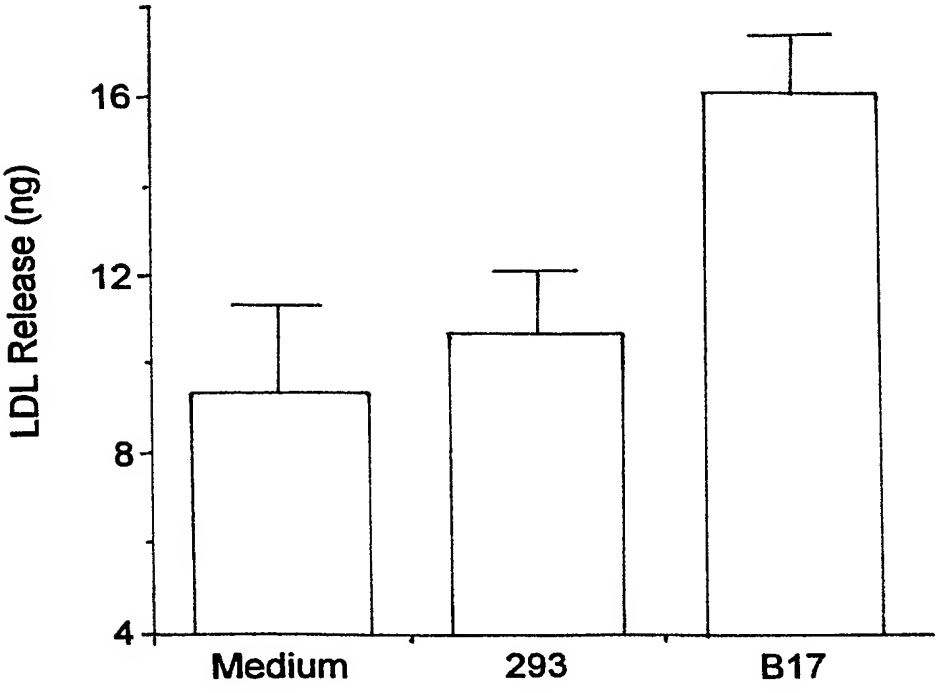

16
12
8
4
LDL Release (ng)
Medium
293
B17

FIG. 9

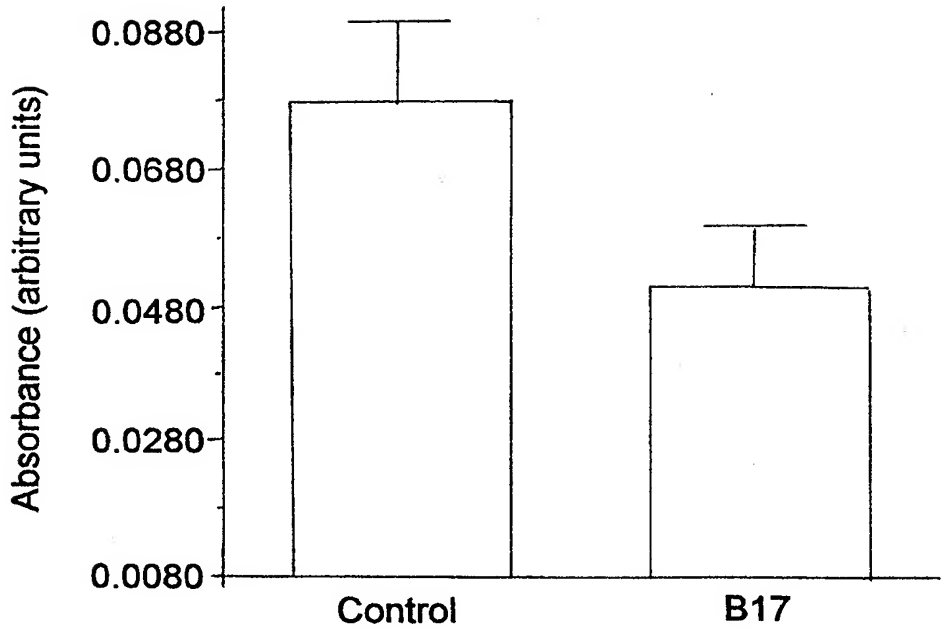

Absorbance (arbitrary units)
0.0880
0.0680
0.0480
0.0280
0.0080
Control
B17

FIG. 10A

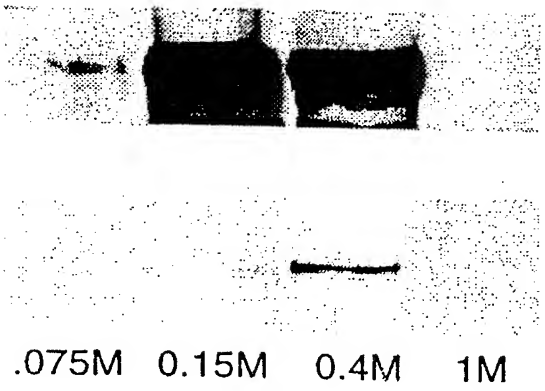
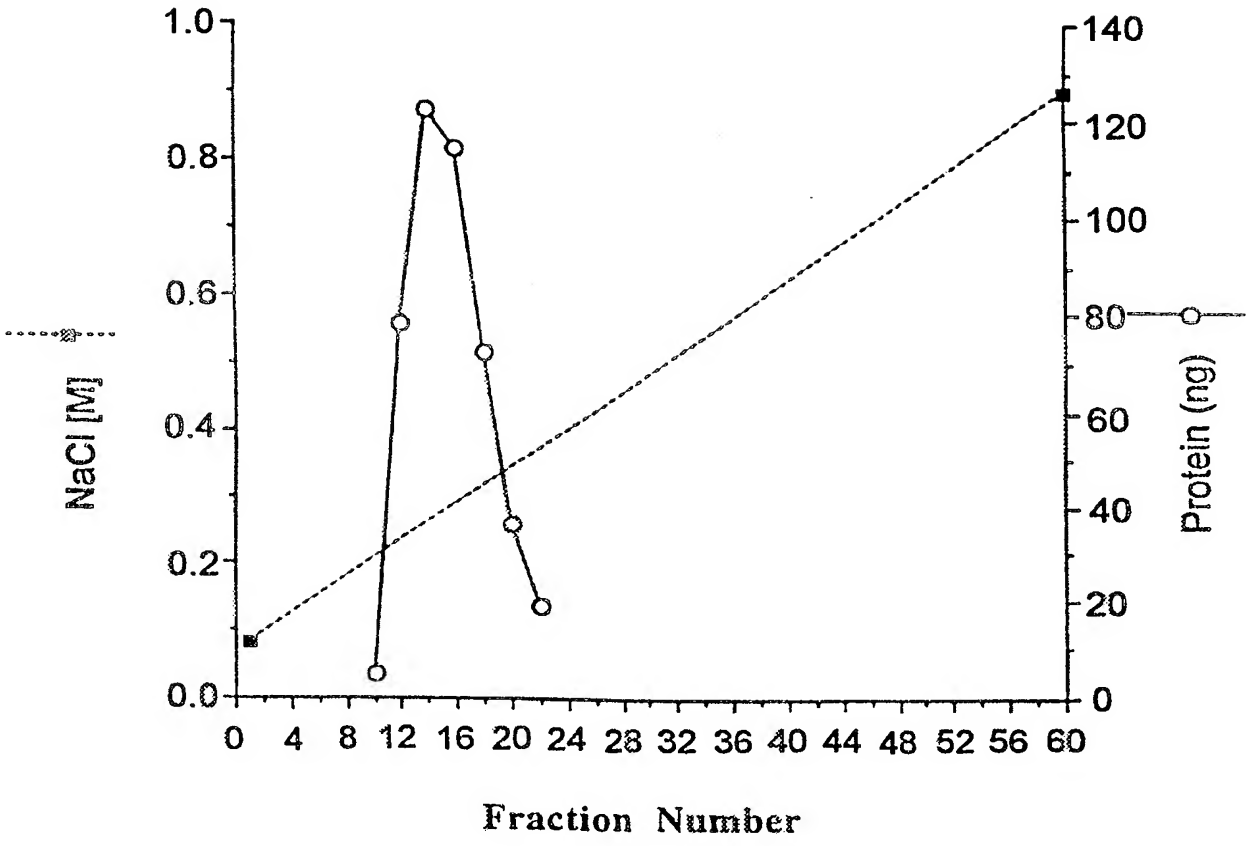


FIG. 10B


1.0
0.8
0.6
0.4
0.2
0.0
NaCl [M]
140
120
100
80
60
40
20
0
Protein (ng)
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60
Fraction Number

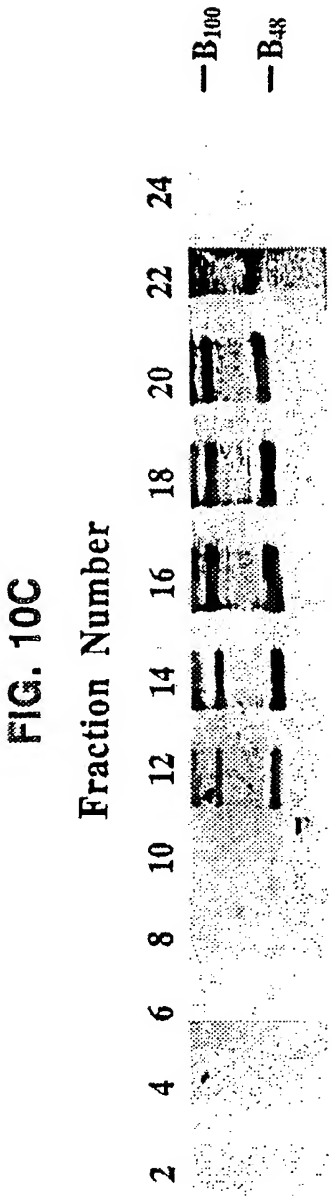


FIG. 10C

FIG. 11A

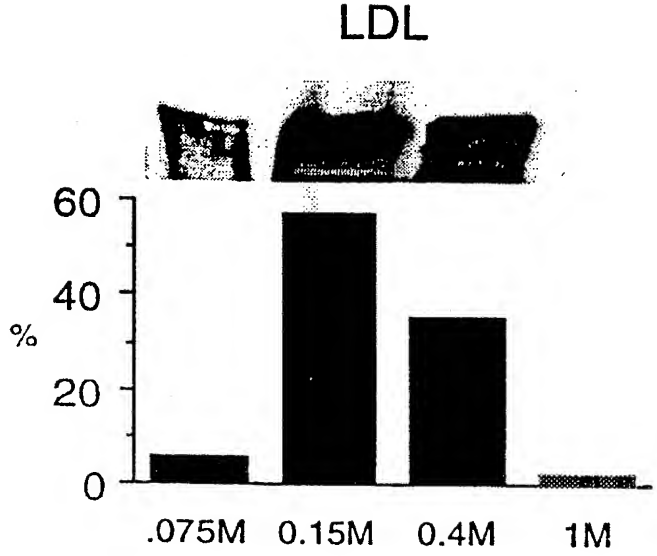


FIG. 11B

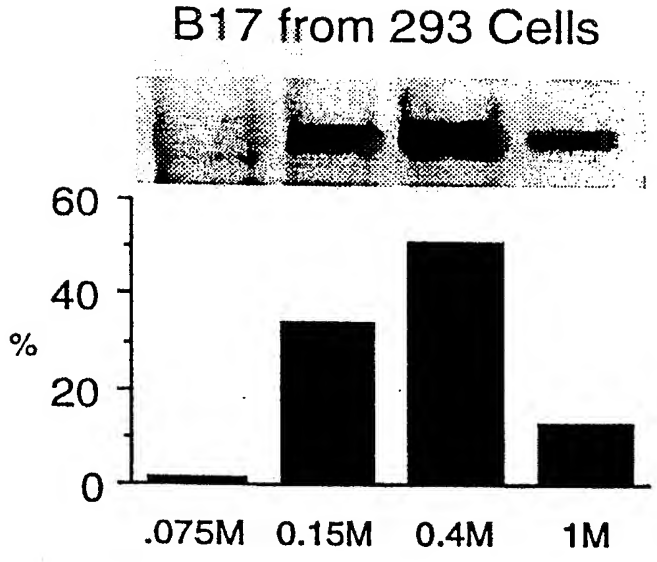


FIG. 12A

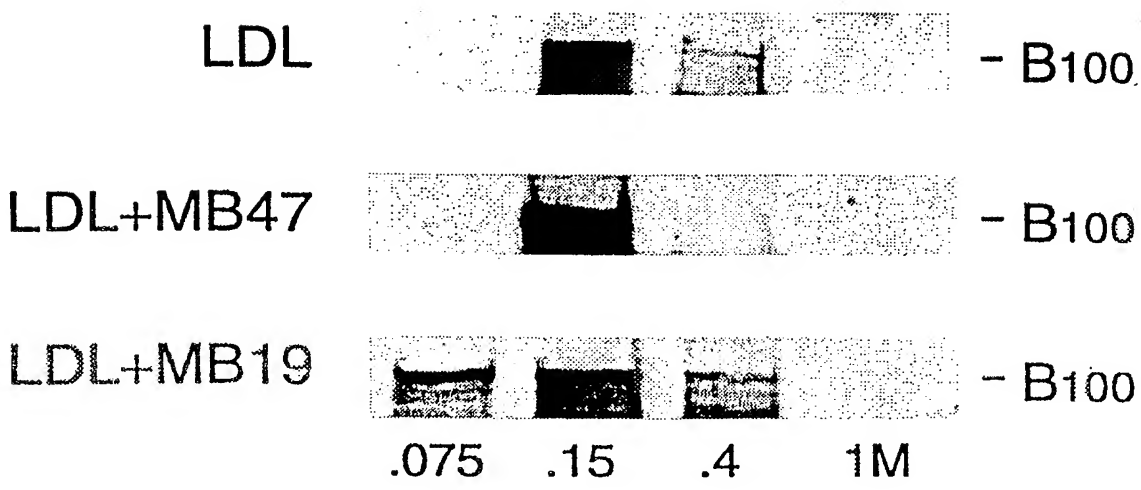


FIG. 12B

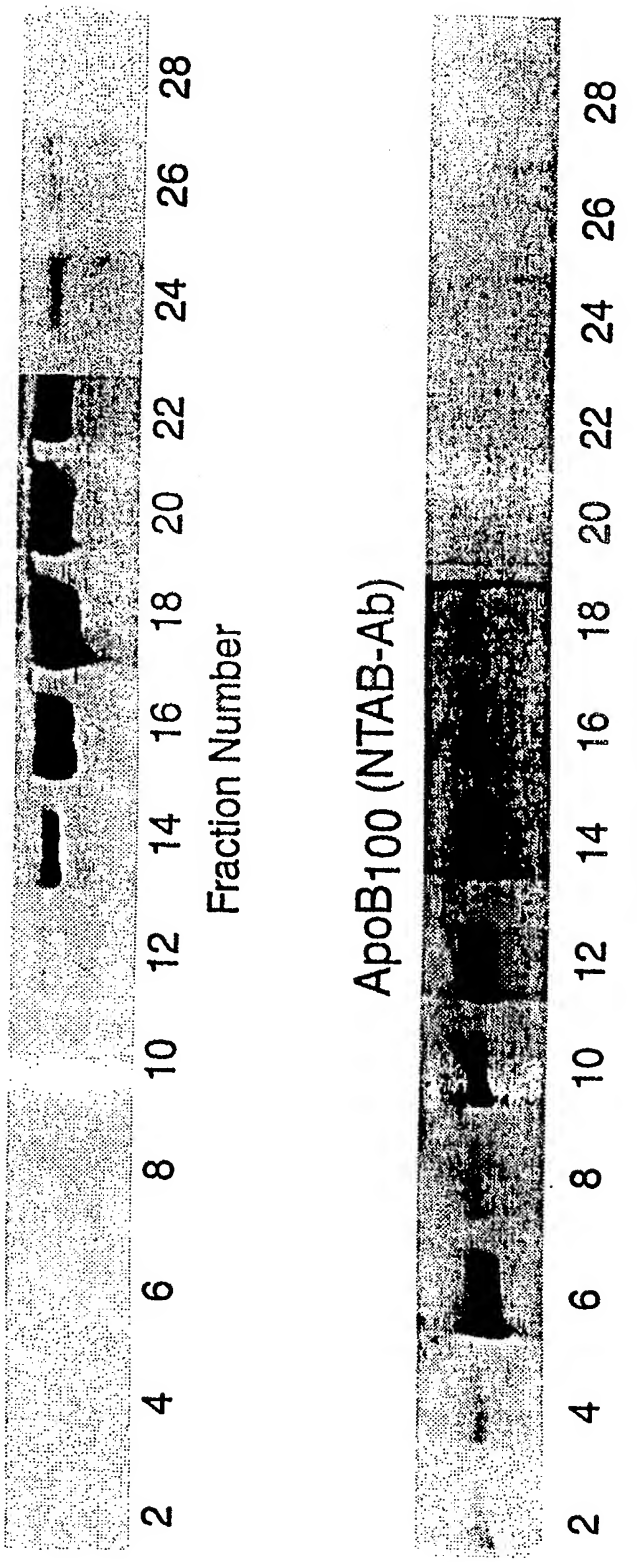

ApoB100 (CTAB-Ab)
ApoB100 (NTAB-Ab)
2 4 6 8 10 12 14 16 18 20 22 24 26 28
Fraction Number

FIG. 13

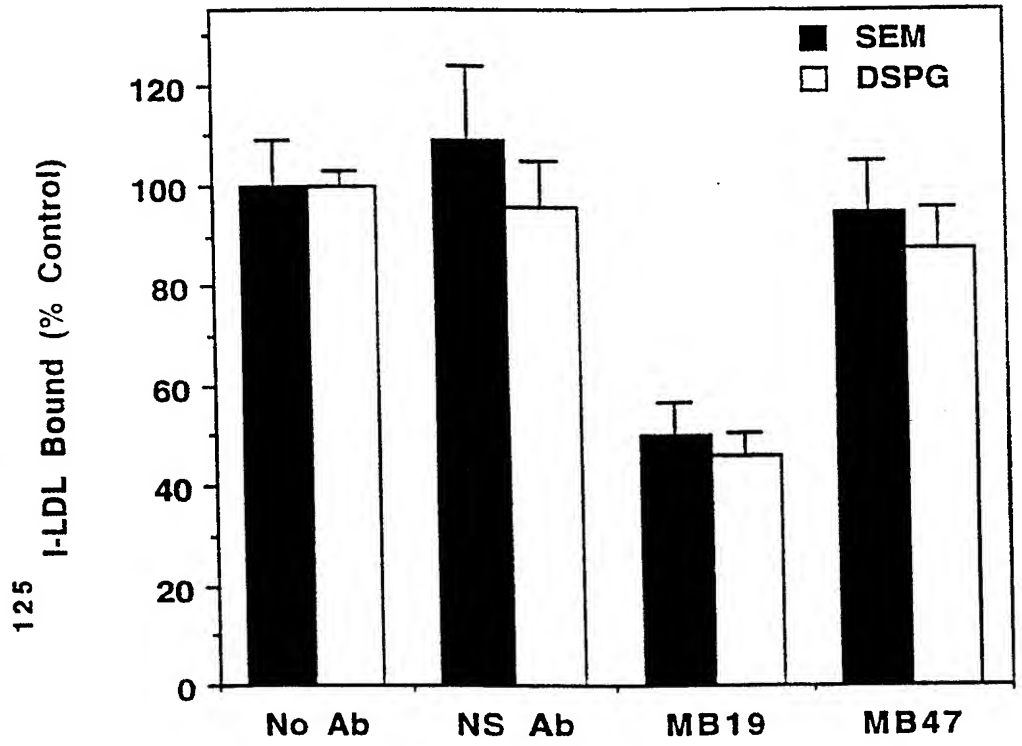

SEM
DSPG
125 I-LDL Bound (% Control)
120
100
80
60
40
20
0
No Ab
NS Ab
MB19
MB47

FIG. 14A

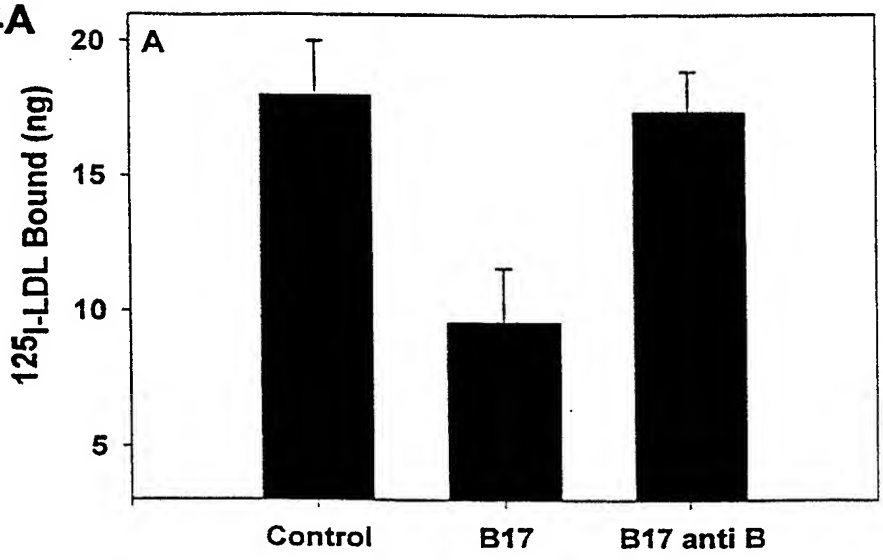


FIG. 14B

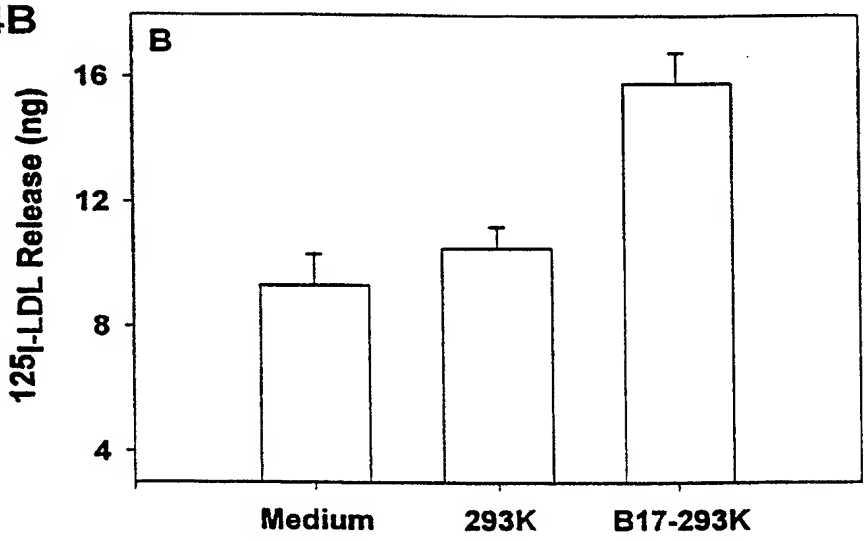


FIG. 14C

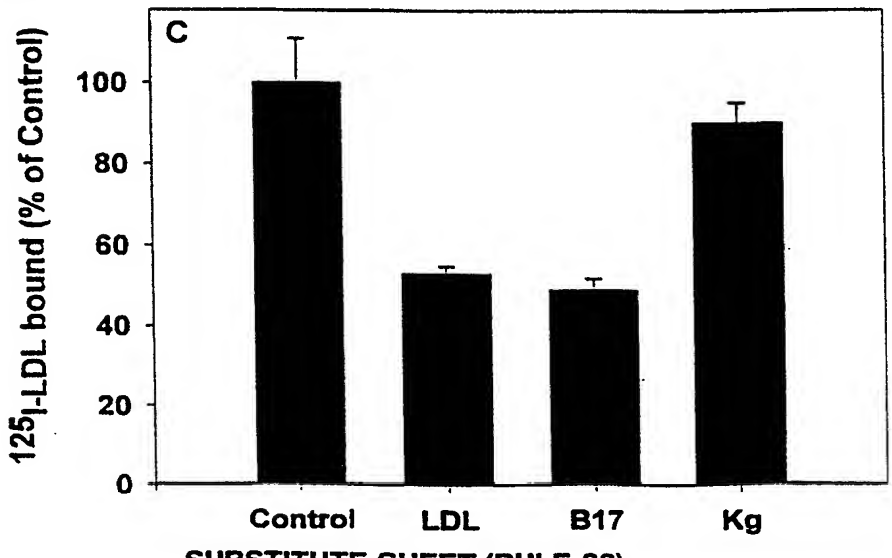
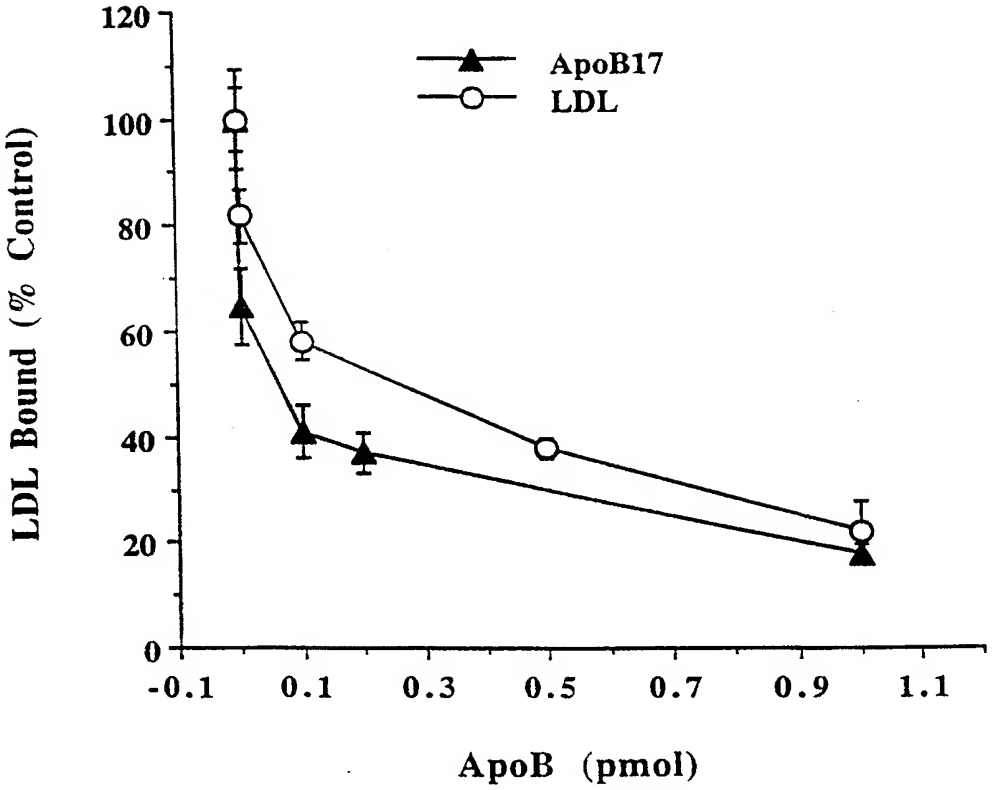


FIG. 15


120
100
80
60
40
20
0
LDL Bound (% Control)
ApoB17
LDL
-0.1
0.1
0.3
0.5
0.7
0.9
1.1
ApoB (pmol)

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: The Trustees of Columbia University in the City of New York

(ii) TITLE OF INVENTION: METHOD FOR INHIBITING THE BINDING OF LOW DENSITY LIPOPROTEIN TO BLOOD VESSEL MATRIX

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Cooper & Dunham LLP
(B) STREET: 1185 Avenue of the Americas
(C) CITY: New York
(D) STATE: NY
(E) COUNTRY: USA
(F) ZIP: 10036

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) INT'L APPL'N NUMBER: NOT YET KNOWN
(B) FILING DATE: Herewith
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: White, John P.
(B) REGISTRATION NUMBER: 28,678
(C) REFERENCE/DOCKET NUMBER: 54086-A-PCT/JPW/AKC

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 212-278-0400
(C) TELEX: 212-391-0526

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 809 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Asp Pro Pro Arg Pro Ala Leu Leu Ala Leu Leu Ala Leu Pro Ala
1 5 10 15

Leu Leu Leu Leu Leu Leu Ala Gly Ala Arg Ala Glu Glu Glu Met Leu
20 25 30

Glu Asn Val Ser Leu Val Cys Pro Lys Asp Ala Thr Arg Phe Lys His
35 40 45

Leu Arg Lys Tyr Thr Tyr Asn Tyr Glu Ala Glu Ser Ser Ser Gly Val
50 55 60

Pro Gly Thr Ala Asp Ser Arg Ser Ala Thr Arg Ile Asn Cys Lys Val
65 70 75 80

Glu Leu Glu Val Pro Gln Leu Cys Ser Phe Ile Leu Lys Thr Ser Gln
85 90 95

Cys Thr Leu Lys Glu Val Tyr Gly Phe Asn Pro Glu Gly Lys Ala Leu
100 105 110

Leu Lys Lys Thr Lys Asn Ser Glu Glu Phe Ala Ala Ala Met Ser Arg
115 120 125

Tyr Glu Leu Lys Leu Ala Ile Pro Glu Gly Lys Gln Val Phe Leu Tyr
130 135 140

```
Pro Glu Lys Asp Glu Pro Thr Tyr Ile Leu Asn Ile Lys Arg Gly Ile
145                 150                 155                 160

Ile Ser Ala Leu Leu Val Pro Pro Glu Thr Glu Glu Ala Lys Gln Val
                165                 170                 175

Leu Phe Leu Asp Thr Val Tyr Gly Asn Cys Ser Thr His Phe Thr Val
            180                 185                 190

Lys Thr Arg Lys Gly Asn Val Ala Thr Glu Ile Ser Thr Glu Arg Asp
        195                 200                 205

Leu Gly Gln Cys Asp Arg Phe Lys Pro Ile Arg Thr Gly Ile Ser Pro
    210                 215                 220

Leu Ala Leu Ile Lys Gly Met Thr Arg Pro Leu Ser Thr Leu Ile Ser
225                 230                 235                 240

Ser Ser Gln Ser Cys Gln Tyr Thr Leu Asp Ala Lys Arg Lys His Val
                245                 250                 255

Ala Glu Ala Ile Cys Lys Glu Gln His Leu Phe Leu Pro Phe Ser Tyr
            260                 265                 270

Asn Asn Lys Tyr Gly Met Val Ala Gln Val Thr Gln Thr Leu Lys Leu
        275                 280                 285

Glu Asp Thr Pro Lys Ile Asn Ser Arg Phe Phe Gly Glu Gly Thr Lys
    290                 295                 300

Lys Met Gly Leu Ala Phe Glu Ser Thr Lys Ser Thr Ser Pro Pro Lys
305                 310                 315                 320

Gln Ala Glu Ala Val Leu Lys Thr Leu Gln Glu Leu Lys Lys Leu Thr
                325                 330                 335

Ile Ser Glu Gln Asn Ile Gln Arg Ala Asn Leu Phe Asn Lys Leu Val
            340                 345                 350

Thr Glu Leu Arg Gly Leu Ser Asp Glu Ala Val Thr Ser Leu Leu Pro
        355                 360                 365
```

Gln Leu Ile Glu Val Ser Ser Pro Ile Thr Leu Gln Ala Leu Val Gln
370 375 380

Cys Gly Gln Pro Gln Cys Ser Thr His Ile Leu Gln Trp Leu Lys Arg
385 390 395 400

Val His Ala Asn Pro Leu Leu Ile Asp Val Val Thr Tyr Leu Val Ala
405 410 415

Leu Ile Pro Glu Pro Ser Ala Gln Gln Leu Arg Glu Ile Phe Asn Met
420 425 430

Ala Arg Asp Gln Arg Ser Arg Ala Thr Leu Tyr Ala Leu Ser His Ala
435 440 445

Val Asn Asn Tyr His Lys Thr Asn Pro Thr Gly Thr Gln Glu Leu Leu
450 455 460

Asp Ile Ala Asn Tyr Leu Met Glu Gln Ile Gln Asp Asp Cys Thr Gly
465 470 475 480

Asp Glu Asp Tyr Thr Tyr Leu Ile Leu Arg Val Ile Gly Asn Met Gly
485 490 495

Gln Thr Met Glu Gln Leu Thr Pro Glu Leu Lys Ser Ser Ile Leu Lys
500 505 510

Cys Val Gln Ser Thr Lys Pro Ser Leu Met Ile Gln Lys Ala Ala Ile
515 520 525

Gln Ala Leu Arg Lys Met Glu Pro Lys Asp Lys Asp Gln Glu Val Leu
530 535 540

Leu Gln Thr Phe Leu Asp Asp Ala Ser Pro Gly Asp Lys Arg Leu Ala
545 550 555 560

Ala Tyr Leu Met Leu Met Arg Ser Pro Ser Gln Ala Asp Ile Asn Lys
565 570 575

Ile Val Gln Ile Leu Pro Trp Glu Gln Asn Glu Gln Val Lys Asn Phe
580 585 590

Val Ala Ser His Ile Ala Asn Ile Leu Asn Ser Glu Glu Leu Asp Ile
595 600 605

Gln Asp Leu Lys Lys Leu Val Lys Glu Ala Leu Lys Glu Ser Gln Leu
610 615 620

Pro Thr Val Met Asp Phe Arg Lys Phe Ser Arg Asn Tyr Gln Leu Tyr
625 630 635 640

Lys Ser Val Ser Leu Pro Ser Leu Asp Pro Ala Ser Ala Lys Ile Glu
645 650 655

Gly Asn Leu Ile Phe Asp Pro Asn Asn Tyr Leu Pro Lys Glu Ser Met
660 665 670

Leu Lys Thr Thr Leu Thr Ala Phe Gly Phe Ala Ser Ala Asp Leu Ile
675 680 685

Glu Ile Gly Leu Glu Gly Lys Gly Phe Glu Pro Thr Leu Glu Ala Leu
690 695 700

Phe Gly Lys Gln Gly Phe Phe Pro Asp Ser Val Asn Lys Ala Leu Tyr
705 710 715 720

Trp Val Asn Gly Gln Val Pro Asp Gly Val Ser Lys Val Leu Val Asp
725 730 735

His Phe Gly Tyr Thr Lys Asp Asp Lys His Glu Gln Asp Met Val Asn
740 745 750

Gly Ile Met Leu Ser Val Glu Lys Leu Ile Lys Asp Leu Lys Ser Lys
755 760 765

Glu Val Pro Glu Ala Arg Ala Tyr Leu Arg Ile Leu Gly Glu Glu Leu
770 775 780

Gly Phe Ala Ser Leu His Asp Leu Gln Leu Leu Gly Lys Leu Leu Leu
785 790 795 800

Met Gly Ala Arg Thr Leu Gln Gly Ile
805

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/21345

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 38/00, 39/395; C12N 15/00
US CL : 424/158.1, 145.1; 514/2, 13; 800/13; 435/4

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/158.1, 145.1; 514/2, 13; 800/13; 435/4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	McCORMICK. S.P. et al. Transgenic Mice Expressing Human ApoB95 and ApoB97. J. Biol. Chem. 19 September 1997. Vol. 272 . No. 38. pages 23616-23622, especially page 23618.	21
Y	PANG. L. et al. Cell-surface expression of an amino-terminal fragment of apolipoprotein B increases lipoprotein lipase binding to cells. J. Biol. Chem. 09 August 1996. Vol. 271. No. 32. pages 19518-19523, especially page 19518.	1-20
Y	SIVARAM. P. et al. An amino-terminal fragment of apolipoprotein B binds to lipoprotein lipase and may facilitate its binding to endothelial cells. J. Biol. Chem. 01 April 1994. Vol. 269. No. 13. pages 9409-9412, especially page 9411.	1-9, 12-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
04 JANUARY 1999

Date of mailing of the international search report
15 JAN 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
ANNE MARIE S. BECKERLEG
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/21345

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG-Medine, Biosis, Cancerlit, Embase, Scisearch, Derwint WPIX, USPATFULL
search terms: apolipoprotein b, apob, apob17, amino terminal or terminus,antibody